# *Detection of chromosomal breakpoints in B-cell neoplasia by interphase fluorescence in situ hybridization on routine paraffin sections*

**Eugenia Haralambieva**

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*Stellingen behorende bij het proefschrift*

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E.Haralambieva, 28 september, 2005

1. Both biological and practical considerations favor fluorescence *in situ* hybridization (FISH) as the preferable technique for targeted detection of translocations in mature B cell lymphomas (this thesis).

2. The application of FISH on routinely processed paraffin tissue samples represents a considerable advance in the application of molecular biology in clinical pathology (this thesis).

3. Chromosomal breakpoints in *MYC* gene are highly associated with Burkitt lymphoma, however they are not a disease specific marker (this thesis).

4. In adult patients, B cell lymphomas with Burkitt-like morphology may often represent transformed NHL (this thesis).

5. Cyclin D1deregulation is an essential oncogenic event in multiple myeloma patients that is triggered by t(11;14)(q13;q32), but also by a yet unknown mechanism often associated with chromosome 11 trisomy (this thesis).

6. Deregulation of essential oncogenes often represents a functional endpoint of various genetic lesions (development).

7. Chromosomal translocations are a hallmark of most B cell lymphomas, however additional genetic and epigenetic events are indispensable in lymphomagenesis (development).

8. I have never let my schooling interfere with my education. Mark Twain

9. No one can make you feel inferior without your consent. Eleanor Roosevelt

10. Life shrinks or expands in proportion to one's courage. Anais Nin

11. All my life I wanted to be something - now I see that I should have been more specific...

RIJKSUNIVERSITEIT GRONINGEN

# Detection of chromosomal breakpoints in B-cell neoplasia by interphase fluorescence in situ hybridization on routine paraffin sections

**Proefschrift**

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door

**Eugenia Haralambieva**

geboren op 14 april 1962
te Sofia, Bulgaria

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# Chapter 1

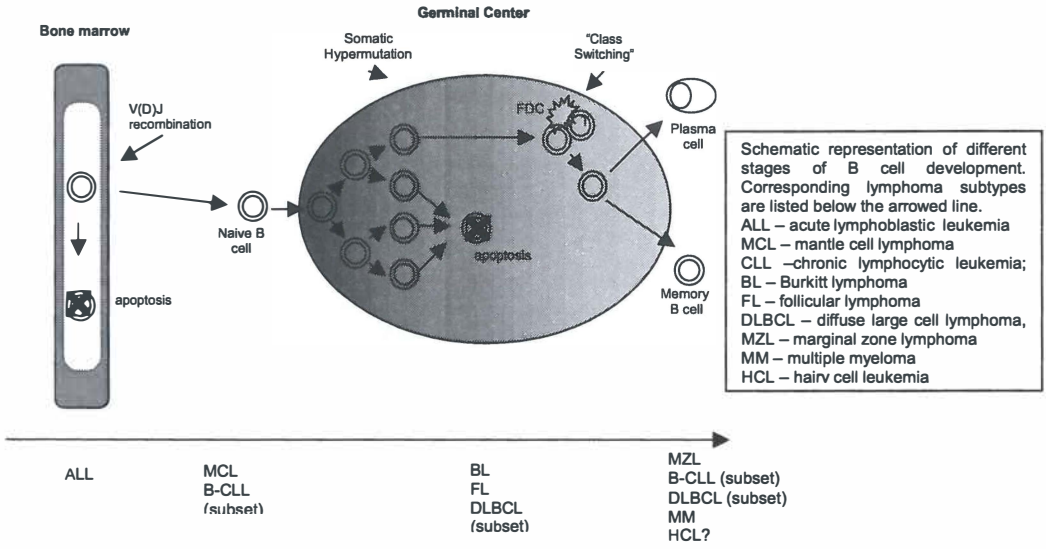
## Introduction

***B cell development and classification and B cell neoplasms***

B cell neoplasms represent a clonal proliferation of B cells and in many respect appear to recapitulate stages of normal B-cell development, ranging from precursor B cells to mature plasma cells.[1,2,3] The capability of B cells to respond specifically to an ultimate variety of antigenic stimuli depends on the expression of an immunoglobulin (Ig) molecule on the cell surface that serves as a B cell antigen receptor (BCR).[3] Therefore, the formation and expression of BCR is a critically important event in the differentiation and maturation of B cells. Apart from BCR expression, this process involves changes in cytomorphology and homing that are correlated to many changes in gene mRNA and protein expression. [3,4] The development of B cell begins within the bone marrow with formation of the hypervariable regions of heavy chain and light chain Ig genes that potentially can recognize foreign antigens by a process called V(D)J recombination. [3,5,6,7] The variable region genes of heavy chain (IgH) are assembled from variable (V), diversity (D) and joining (J) elements and those of the light chain (κ or λ) by V and J elements. In the germline configuration there are many different V, D and J elements (for the IgH gene, respectively 40-46 VH, 25 DH and 6 JH elements). Upon rearrangement in precursor B-cells there is an almost random assembly of these elements with deletion of the DNA located between them, and additional nucleotide insertion, resulting is an almost indefinitive number of possibilities, for all Ig loci estimated at $>10^{12}$ . Each precursor B cell generates a particular set of genes that encodes a distinct Ig molecule. Only these B cells that acquire heavy- and light chain variable regions genes that can be translated into protein (dependent on a correct reading frame) survive, leave the bone marrow and form mature naïve B cells. Cells with out-of-frame rearrangement die by programmed cell death. B-cells, expressing auto-reactive receptors either are also eventually removed by apoptosis or undergo secondary V(D)J rearrangements. Naïve circulating B cells can be recruited into the germinal centers (GC) of secondary lymphoid organs[8], i.e. lymph nodes, the spleen, and mucosa associated lymphoid tissue (MALT), where during the course of a T cell dependent immune response they undergo further genomic modification of their BCR by somatic hypermutation and class switch recombination (CSR). Somatic hypermutation is a process by which mutations (mainly single nucleotide exchanges, but also deletions and duplication) are inserted into (mainly) the variable region of the Ig genes.[9,10,11] As a result, antibody variants with different affinities to the antigen are generated, and cells expressing BCR with a relatively high affinity to the respective antigen are positively selected. GC B cells undergo several rounds of proliferation and selection, and eventually the cells expressing favorable mutants leave the GC and differentiate into long lived plasma cells or memory B cells. However, by far most GC B cells acquire unfavorable mutations and undergo apoptosis, a process which is facilitated by the temporary downregulation of the anti-apoptotic bcl-2 protein. Besides this process of affinity maturation, a fraction of GC B cells is submitted to immunoglobulin class switch recombination with respective changes of the isotype of the expressed BCR (from IgM/IgD to IgG, IgA or IgE). CSR results in different effector functions of the antibody for instance because of the different complement binding characteristics of the isotypes.[12,13] At the molecular level, Cμ and Cδ genes that are originally expressed by all naïve B cells are replaced by a Cγ, Cα or Cε gene a recombination event that deletes the DNA between repeated sequences (called switch regions) located upstream of each constant gene. In consequence, both the mutation status of the variable heavy chain genes as well as the isotype expression of the lymphoma cells represent useful markers of the different stages of B cell development [3,5] : the presence of 'ongoing' mutations that vary between lymphoma cells but are clonally related, indicates a GC origin, a homogeneous mutational status of the variable heavy-chain region denotes a post-GC origin, and the lack of any mutations assumes a pre-GC stage of the tumor (see Figure 1).

The recently published World Health Organization (WHO) classification of non Hodgkin's lymphoma (NHL) represents a significant advance in our ability to understand, identify and therefore, treat different specific lymphoma entities. It is based on the concept of the clinicopathologic entities in which histology, immunophenotype, (molecular) genetic data as well as clinical features are integrated, but also taking into account the putative cell of origin of different NHL.[1]

**Figure 1. B-cell development and corresponding B-cell NHL**




An excellent example of this successful approach is the current diagnosis and classification of mantle cell lymphoma (MCL) with a morphology and immunophenotype corresponding to its origin of naïve B-cells of the follicular mantle zone.[1,14,15] The translocation t(11;14)(q13;q32) that results in cyclin D1 expression represents a genetic hallmark and the disease. The median age of patients is about 65 years with a marked male preponderance and a poor clinical outcome with a median overall survival of 3.0 to 3.5 years.

In spite of all recent research in the field, the normal cell counterpart of some entities remains to be defined- e.g. the corresponding putative cell of origin of hairy cell leukemia (HCL) has not been identified, although most data suggest a late postgerminal center derivation of the tumor cells.[16,17] These investigations also revealed some unexpected findings, for instance recent reports indicate that about 50% of the B-cell chronic lymphocytic leukemia (B-CLL) cases carry unmutated IgV genes and derive from naïve B cells, while the rest of the CLL cases resemble memory B cells with mutated IgV genes. Gene expression profiling of CLL indicates a rather homogeneous phenotype related to memory B-cells, however, with some essential differences in BCR signaling between the two subtypes. Importantly, patients with mutated and unmutated CLL have a significantly different clinical outcome.[18-22]

Diffuse large B-cell lymphoma (DLBCL) is also a heterogeneous entity not only in morphology, immunophenotype and genetics, but also in respect to the gene expression profile and the presumptive cell of origin. There are at least 2 and possibly more, molecular subtypes: lymphomas with a germinal center B cell-like profile of gene expression (GCB profile), that resemble reactive germinal center cells, and lymphomas that express genes more related to activated B-cells (ABC profile) of post germinal center origin.[23-26]

***Chromosomal translocations in B-non-Hodgkin's lymphoma***

During the past decade, extensive data have been collected indicating the association of certain recurrent, sometimes entity defining genetic abnormalities with different hematopoietic neoplasias.[1, 5, 27-31] Many of these abnormalities include chromosomal translocations, deletions, amplifications and mutations which are involved in the pathogenesis of the disease through mechanisms including activation of a proto-oncogene, disruption of a tumor suppressor gene or generation of a fusion gene with "gain of function" pro-oncogenic activity. In most mature B-cell neoplasms chromosomal translocations result in the transcriptional deregulation of an oncogene by juxtaposing it to regulatory sequences of genes that are constitutively expressed in mature B cells, most often the Ig genes (Table 2, Figure 2).[5, 27, 31]

The important oncogenes described up to date include BCL1/*CCND1* at 11q13 involved in mantle cell lymphoma (MCL) and some multiple myelomas (MM), *BCL2* at 18q21 rearranged in almost all follicular lymphoma (FL) and some DLBCL, c-*MYC* at 8q24 involved in almost all Burkitt's lymphoma (BL), few

DLBCL, and rare cases of (transformed) FL as well as clinical accelerated MM, *BCL6* at 3q27 involved in more than on third of DLBCL and few FL, *MALT1* on 18q21, rearranged in variable percentage of MALT lymphomas, *CCDN3* on 6p21, *FGFR3/MMSET* on 4p16, c- *maf* in 16q23 rearranged in MM [31,32, 59-61] (see Table 1).

***Table 1 Non-random chromosomal translocations in mature B neoplasms***

| *Chromosomal translocation* | *Gene* | *function* | *B-NHL subcategory* | *frequency* | *references* |
|---|---|---|---|---|---|
| *t(14;18)(q32;q21) and variants* | *BCL2* | *anti-apoptotic protein* | *FL<br>DLBCL<br>CLL* | *80-90%<br>20-30%<br><1%* | 1,35-37 |
| *t(1;22)(q22;q11)* | *FCGR2B* | *low affinity Ig Fc receptor* | *FL, DLBCL* | *<1%* | *62-64* |
| *t(8;14)(q24;q32) and variants* | *MYC* | *transcription factor* | *BL<br>DLBCL<br>FL<br>MM* | *~100%<br>5-10%<br><1%* | 1,31,32,35,37-46 |
| *t(3;14)(q27;q32) and variants* | *BCL6* | *transcriptional repressor* | *DLBCL<br>FL* | *40%<br>?%* | 1,35,50-55 |
| *t(11;14)(q13;q32)* | *CCND1* | *G1 cyclin* | *MCL<br>MM* | *>95%<br>~20%* | 1,14,31,32 |
| *t(9;14)(p13;q32)* | *PAX5* | *transcription factor* | *variant SLL* | *not established* | *49* |
| *t(11;18)(q21;q21)* | *API2/ MALT1* | *anti-apoptosis protein* | *MALT* | *~30%** | *56-58* |
| *t(14;15)(q32;q11-13)* | *BCL8* | | *DLBCL* | *~4%* | *65* |
| *t(1;14)(q21;q32)* | *MUC1* | *cell surface receptors* | *DLBCL* | *<1%* | *66* |
| *t(1;14)(p22;q32)* | *BCL10* | *apoptosis regulatory protein* | *MALT* | | *67* |
| *t(14;18)(q32;q21)* | *MALT1* | | *MALT* | *~20%** | [68] |
| *t(14;19)(q32;q13)* | *BCL3* | *IκB* | *CLL/SLL* | *<5%* | *69* |
| *t(4;14)(p16;q32)* | *FGFR3/MMSET* | | *MM* | *15-20%* | *59* |
| *t(14;16)(q32;q23) and variant* | *c-maf* | *transcription factor* | *MM* | *2-10%* | *60* |
| *t(6;14)(p21;q32)* | *CyclinD3* | *Cell cycle protein* | *MM* | *3-4%* | *61* |
| *t(6;14)(p25;q32)* | *MUM1/IRF-4* | *interferon regulatory factor* | *MM* | *not established* | *70* |
| *der7q21* | *CDK6* | *cell cycle kinase* | *splenic MZL* | *60-70%* | *71,72* |

FL – Follicular lymphoma; DLBCL – diffuse large B cell lymphoma; CLL – chronic lymphocytic leukemia; BL – Burkitt lymphoma; MM – multiple myeloma; MALT – mucosa-associated lymphoid tissue lymphoma; SLL – small lymphocytic lymphoma; MZL – marginal zone lymphoma

Three enhancers are known to regulate IgH expression in B cells and subsequently are involved in a translocation event leading to oncogene deregulation: the intronic enhancer (Eμ) located in the intron between the JH and switch μ sequences and the two 3' IgH enhancers downstream of the alpha genes (Eα1 and Eα2). In case of a break in the IgH locus, these enhancers become differently positioned: in case of a breakpoint in the V,D or J elements, all three enhancers are juxtaposed to the oncogene; in case of a breakpoint in Sμ both Eα1 and Eα2 are juxtaposed and in case of a break in many downstream constant genes only Eα2 is juxtaposed to the gene. Thus, in case of a reciprocal translocation within a switch region these enhancers become dissociated on each derivative chromosome, therefore (onco)genes on both alleles may become deregulated by such a translocation event.[5, 73,74] Similarly to the IgH enhancers, Ig κ and λ light chain genes are also involved in chromosomal translocations, although with a relatively low frequency. Translocations resulting in the generation of a fusion gene are more common in leukemias and in T cell lymphomas [75] with two exceptions in mature B cell lymphoma: the t(11;18)(q21;q21) with an API/MALT1[58]and t(2;17)(p23;q23) with clathrin/ALK fusion product [76](Figure 2).

**Figure 2. Model of chromosomal translocations**

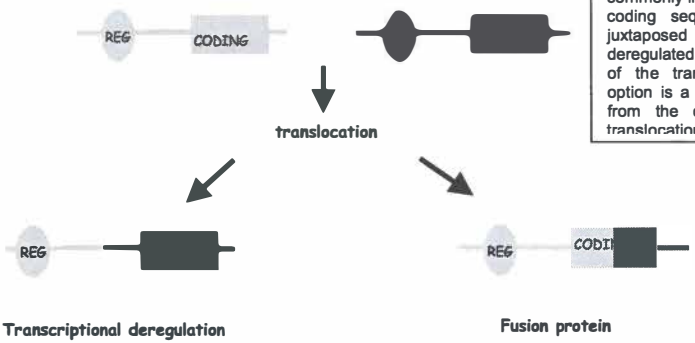



Figure 2 illustrated two models of chromosomal translocation. Most commonly in mature B cell neoplasm the coding sequences of one gene are juxtaposed and subsequently deregulated by the regulatory sequences of the translocation partner. Another option is a generation of a fusion gene from the coding sequences of both translocation partners

The pathogenetic role of chromosomal translocations is demonstrated by *in vitro* transformation studies and experiments in transgenic animal model. [77-82] These experimental models indicate that chromosomal translocations contribute to lymphoma development, however, are not sufficient to cause it, which is consistent with the general requirement of multiple genetic lesions in tumorigenesis. In line with this view, some of the most common translocations in B cell lymphoma have also been detected as rare somatically acquired events in healthy volunteers. [83] The mechanisms by which chromosomal translocations occur are poorly understood, although with respect to the Ig loci, they appear to be associated with a dysfunction of the genetic remodeling mechanisms operating in normal lymphoid cells, including the Ig gene rearrangements (VDJ and class switch recombination) and somatic hypermutation.[5] Correspondingly, different chromosomal translocations occur at different stages of B-cell development. For example, the breakpoints of the t(14;18) in FL, t(11;14) in MCL and t(1;14) in MALT show structural features suggesting a generation from an aberrant VDJ recombination attempt in bone marrow precursor B cells.[5,84-86] Breakpoints in most IgH translocations in MM, 3q27 translocations in DLBCL and t(8;14) in sporadic BL are located within Ig switch regions, therefore, they likely have been generated by aberrant isotype switching [5,31,41, 49, 87-92] in germinal center B cells. The location of certain translocation breakpoints {e.g. t(8;14 ) in endemic BL, some t(3;14) in DLBCL [5, 41]} in already rearranged VDJ genes that contain somatic hypermutations, suggests that they are mediated by an aberrant somatic hypermutation machinery during the germinal center reaction.

***Detection of the chromosomal translocation***

Conventional cytogenetic analysis represents a "gold standard" for detection of recurrent genetic lesions and in addition provides an overview on the complete karyotype.[36, 93, 94] However, it is laborious and time consuming and requires viable, dividing cells from fresh tissue samples. Its rate of success largely varies depending on the availability of proliferating tumor cells and the quality and number of the metaphase chromosomes. Of note, there may be a selective growth advantage of residual normal cells in the culture which may dilute out the clone and hence the relevant karyotypic abnormality. In addition, many tumors have numerous and complex chromosomal rearrangements, including non-reciprocal rearrangements that complicate the identification of specific chromosomes and diagnostic abnormalities. Often rearrangements involve chromosomal bands of similar size and staining patterns (e.g. t(12;21)(p12;q21) in pediatic acute lymphoblastic leukemia)[95] or that are poorly stained (e.g. subtelomeric regions) and therefore invisible by standard G-banding. Thus, some important recurrent translocations were selectively overlooked in conventional karyotypes, for instance the t(4;14)/FGFR3/MUMSET and t(14;16)/*c-maf* in MM,[56,60,62] as well as many 3q27/*BCL6* translocations in DLBCL and FL.[55]

Molecular methods as Southern blot and the polymerase chain reaction (PCR) represent important tools for detection of chromosomal translocations in B-NHL.[32,96-99] PCR has the advantage of speed and sensitivity, and needs very little patient's material. At the genomic level it can be performed if both chromosomal partners are known and the breakpoints are clustered. In case of a fusion transcript like the t(11;18) in MALT lymphomas, the translocation can be much easier detected at the RNA level by RT-PCR. PCR detection at the genomic level is severely hampered by the lack of local clustering in many NHL translocations (e.g. the BCL1/ *CCND1* and *MYC* translocation breakpoints are dispersed over regions of

more than 300 kb)[100-105] and by the fact that some genes involved in translocations (e.g. *BCL6*) can be juxtaposed to multiple translocation partners. [106,107] Therefore, to reach a detection rate that is acceptable for diagnostic settings, multiple primer pairs and more sophisticated long distance PCR protocols are needed. [42, 91, 99] Many clustered breakpoints are relatively easy to detect by Southern blot analysis and restriction length analysis (e.g. MBR/*BCL6* breakpoints in DLBCL), however others (e.g. *MYC* breakpoints in most endemic Burkitt lymphomas) [102,103] extend beyond the length of the DNA fragments generated by the restriction enzymes and labeled by the genomic probes. Therefore, the use PCR and Southern blot analysis for regular detection of the translocations in B cell neoplasia is difficult and complex in the majority of the cases. As an alternative, pulsed-field gel electrophoresis (PFGE) has been used to detect breakpoints, however, this technically demanding method is not applicable in a diagnostic setting.

In some instances, an abnormal expression pattern of the RNA or protein product strongly correlates with the presence of an underlying genetic lesion. Aberrant gene expression on the RNA level may then be quantified by real-time PCR or microarray gene expression studies. [108,109] An example is the detection of cyclin D1 by RT-PCR in leukemic cells of mantle cell lymphoma, used as a diagnostic tool by especially hematologists. Overexpression of the gene product can be detected by conventional immunohistochemistry as well.[110]

This latter approach as used by most pathologists has the major advantage that is easily applicable in a routine pathology setting and that it also provides information about the tissue distribution, as well as the cellular and subcellular localization of the oncogenic proteins. Some conditions facilitate the use of these assays: it is especially useful in cases where the gene involved in the chromosomal rearrangement is not expressed by the normal haematopoietic cells. This is the case for cyclin D1 and ALK. [111] Other proteins have a restricted expression pattern in normal cells (e.g. BCL2 labels mantle zone B cells and T cells but not the normal germinal centres) and in consequence their abnormal distribution may indicate the presence of a genetic lesion and malignancy (e.g. BCL2 labeling of the neoplastic follicles of FL).[112,113] In other cases an altered subcellular localization of the gene product might hint to a specific translocation: wild type BLC10 is localized in the cytoplasm but aberrant nuclear BCL10 expression in MALT lymphomas[114] is associated with t(1;14) and t(11;18) translocations, both resulting in NF-κB activation. Similarly, different patterns of ALK protein expression in the tumor cells (cytoplasmic and nuclear, versus granular, membranous or an other localization) hint at the involvement of specific fusion partners of ALK [115] In contrast, immunocytochemical studies of widely expressed gene products (e.g. MYC)[110] generally have little clinical value. Thus, in some diseases there is a strong correlation between the underlying genetic lesion and aberrant gene expression (e.g. cyclin D1 expression and t(11;14) in MCL) [116,117] making the method valuable in regular diagnostic practice, in other diseases the use is restricted to certain considerations of differential diagnoses (e.g. BCL2 expression and t(14;18) in FL versus follicular hyperplasia) [1,33, 113], while there is no such correlation in many other diseases such as DLBCL. Therefore, breakpoint analysis can be complemented but not simply replaced by RT-PCR or immunohistochemistry for the respective oncogene products and gene expression should be interpreted in the context of the individual malignancy. Although BCL2 expression is not directly correlated with t(14;18) in DLBCL, recent data indicate that the genetic lesions and the expression of the respective protein and RNA might be of interest for their prognostic value. Thus, BCL2 expression in the context of a t(14;18) in DLBCL confers a germinal center B cell-like profile of gene expression (GCB profile) and correspondingly a favorable outcome, while BCL2 expression without a t(14;18) is more related to NF-kB activation in activated B-cells (ABC profile) and a poor survival. [35, 118-120]

***Fluorescence in situ hybridization - general principles and application***

Fluorescence *in situ* hybridization (FISH) is a relatively simple technique that allows targeted detection of both numerical and defined structural chromosomal abnormalities and the technique also provides information at the single-cell level.[121-124] FISH is based on the same principle as Southern blot analysis, namely the ability of single-stranded test DNA to anneal to complementary target DNA sequences. The test DNA, referred to as a probe, is labeled through incorporation of reporter molecules and the reaction is 'in situ' visualized and localized by direct or indirect immunofluorescence. FISH can be applied on intact chromosomes of metaphase cell preparations, interphase nuclei obtained from imprints, cell suspensions, frozen tissue or paraffin tissue blocks, on already cut paraffin tissue sections, or on isolated DNA molecules, the latter mostly called DNA fibre FISH.

As a cytogenetic method, FISH is performed on metaphase preparations supplementary to banding analysis with a superior sensitivity for detection of both numerical and structural aberrations. Different types of probes can be used, i.e. whole chromosome probes (chromosome painting), centromeric probes or locus specific probes. Due to the high degree of DNA condensation, the resolution of metaphase FISH is 2-3 Mb and small size probes can be used with acceptable efficiency. Multicolor metaphase FISH (M-FISH/SKY) [125,126] utilizes differentially labeled whole chromosome probes for each individual chromosome or each chromosomal arm and thereby provides simultaneous information over the complete karyotype. This method dramatically improves the sensitivity of the conventional banding analysis. It is valuable for identification of structural abnormalities that are too small to be detected by conventional cytogenetics such as insertions and characterization of marker chromosomes and/or complex karyotypes. However, the method shares all

limitations of the banding analysis, e.g. the need of viable dividing target cells and their possible selection due to a growth disadvantage, and the method also needs well stretched chromosomes to allow a reasonable resolution.

Fibre FISH is a molecular method applied on mechanically stretched DNA molecules that are isolated from fresh or frozen cell and/or tissue samples and immobilized on glass object slides.[127, 128] The method is useful for fine mapping of the deletions, insertions and chromosomal translocations at a level of 5 to several hundreds kilobases, but due to the technical difficulties the method is not useful as a diagnostic tool in daily practice.

Interphase FISH provides a unique possibility for targeted detection and quantification of defined numerical and structural aberrations in non-dividing or terminally differentiated cells. Since the technique is relatively rapid and straightforward, with a high sensitivity and specificity, different interphase FISH strategies have been developed and extensively applied on nuclear suspensions of fresh or frozen cell and/or tissue samples and cytological imprints. [121-124] The regular use on routine paraffin embedded tissue samples is impaired by technical problems (e.g. weak or /and inconsistent hybridization signal, low signal to noise ratio, ambiguities in interpreting the results), and therefore, its use has mostly been restricted to the detection of numerical chromosomal aberrations in solid cancer. The variable success rates may be related to a combination of factors including the small size of the targets, their degradation and / or masking during fixation and the further processing of the sample as well as the use of a relatively insensitive detection systems and fluorescence microscope equipment. More recently, we and other groups reported on the successful application of this method on nuclei isolated from paraffin tissue blocks and/or paraffin tissue sections.[129-136]

***Interphase FISH on formalin fixed, paraffin tissue samples – technical and methodological considerations***

The introduction of the interphase FISH protocols for paraffin tissue samples offers an important tool for detection of chromosomal aberrations, since these methods allow the use of samples that are not selected by the restricted availability of fresh or frozen tissue. This refers to biopsy samples from distant and/ or small laboratories where storage and transportation of frozen tissue is difficult, retrospective and multicentre studies, referral cases, serial samples from relatively rare disorders, and the increasing number of small biopsies (endoscopic, needle, stereotactic) that are entirely fixed for diagnostic purpose.

To obtain consistent, reproducible hybridization signals on fixed, paraffin embedded tissue samples various FISH approaches have been used. One option is the implementation of probes annealing to large repetitive DNA sequences, resulting in strong and easily detectable signals.[130,131, 133,137,138] Indeed, probes for repetitive α -satellite peri-centromeric DNA targets have been extensively used for the detection of numerical aberrations on archived samples, but also in combination with a locus specific probe to detect chromosomal breakpoints.[130,131,138] Alternatively, large insert clones such as multi-yeast artificial chromosome (YAC) constructs (with a total length of hundreds of kb's to one or more Mb) have been applied as locus specific probes.[133, 137] Both the use of peri-centromeric probes and large multi-YAC probes have important disadvantages. The pericentromeric regions are mostly far away from the breakpoint under study, resulting in considerable distances between the signal and studied target in the relatively decondensed DNA of interphase nuclei. YAC probes cover large regions of DNA that contain much "junk" DNA full of partially repetitive DNA sequences. These sequences cause a high background upon hybridization, while if appropriately blocked reduce the intensity of the specific signal.

In general, various factors such the physical distance between the probes, degree of chromatin condensation and molecular structure of the region will be of influence on the FISH signals, and especially will determine whether FISH signals generated by two probes will co-localize or segregate in interphase nuclei. Thus, segregation and co-localization of distant probes as mentioned above might be relatively fortuitous in interphase nuclei and give rise to ambiguities in the interpretation of the results.[138] Furthermore, neither of these approaches do allow exact localization of the breakpoint, which is increasingly important since some genomic areas contain a high density of genes that all might be involved in a translocation. A different option to the use of these probes annealing to large DNA targets, is the development of optimized FISH protocols for fixed, paraffin embedded tissue samples that integrate different pre-treatment and denaturation steps, in combination with sensitive detection systems and software. This approach allows the use of relatively small probes devoid of repetitive sequences that directly target the region of interest.

For detection of translocation breakpoints in interphase nuclei, various FISH strategies have been used [123] as schematically represented in Figure 3. Single probes spanning the entire breakpoint region that split in case of a translocation breakage and, therefore, generate an additional signal have been used.[139] However, this approach always needs to exclude polysomy. Furthermore, the size of the additional signal generated by the break depends on the exact position of the breakpoint and in some instances it can be very small and difficult to visualize. Finally, in approximately 10-20% of all translocations, the breakpoint is accompanied by deletion of the telomeric part, generating false negative results.

Two color FISH breakpoint analysis [120-124] can be performed using a so-called co-localization or segregation assay. The co-localization assay makes use of probes that are specific for the two different loci

that are randomly localized in normal nuclei but co-localize upon a translocation event and juxtaposition of the two loci (Figure 3). The segregation assay makes use of two breakpoint flanking signals that co-localize in normal nuclei but segregate upon chromosomal breakage (Figure 3).

Both practical and biological considerations are in favor of the segregation assay over the co-localization assay. First, due to the fortuitous overlap of signals in normal cells, the cut-off levels are generally much higher in FISH co-localization assays than in segregation assays.[140] Secondly, some authors described a non-random distribution of the genes/loci that are involved in translocations in normal nuclei further increasing the chance of co-localization in normal cells. [141-143] Thirdly many target genes involved in translocation (e.g. *MYC, BCL6*) [41,106,107] have variable translocation partners and in case of a co-localization assay probes for all possible partners should be applied. Finally, in tissue sections both the cutting artifacts as well as the overlap of nuclei generate loss as well as an increase of signals, the latter by accidental overlap. In case of a co-localization assay and/or the use of breakpoint spanning probes, these patterns are difficult to recognize and interpret. This complexity is even increased in case of additional numerical abnormalities such as trisomy, which is not uncommon in cancer. More complex FISH approaches, representing different combinations of the above described, significantly improve the specificity of the method, [123] however, they may result in complex patterns that in some instances are very difficult to analyze.

**Figure 3 Interphase FISH approaches**

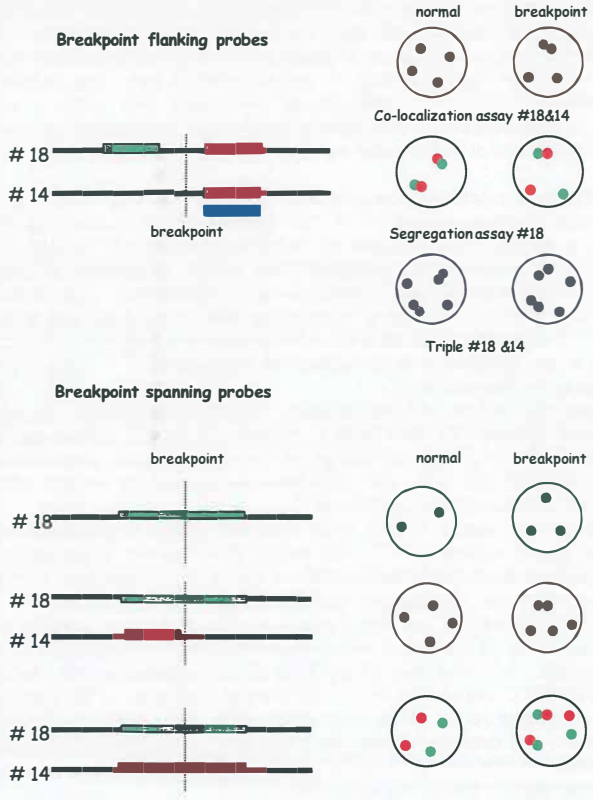



Figure 3 illustrated different intephase FISH approaches. On the left sides are present schematically the locations of the FISH probes into the genomic regions of interest. On the right side are demonstrated different FISH patents produced by the probe in the normal nuclei and upon the translocation break.

Chromosomal breakpoints in fixed, archived material can be studied by interphase FISH using routine paraffin tissue sections or nuclear preparations, isolated from paraffin tissue blocks. The major advantages of FISH on paraffin tissue sections are that the translocation carrying cells can be analyzed in their histological context and that the method needs less patients' material. In contrast, the use of isolated nuclear preparations [135,136] avoids the artifacts generated by truncation and overlapping of the cell nuclei in tissue sections.

Therefore, the scoring and interpretation of the hybridization signals is less complex and the method is preferable for detection of numerical aberrations or in case of a combination of structural and numerical abnormalities of the targets. However, there is little control on the distribution and number of tumor nuclei in

the specimen during the procedure of isolating nuclei from tissue blocks, while FISH on intact paraffin tissue sections allows a more supervised analysis of chromosomal aberrations.

There are few data available about possible procedures for evaluation and interpretation of the FISH results in paraffin embedded tissue samples. One option is to adopt the scoring procedures and statistical methods regularly used for fresh or frozen cell preparations [144] However, in tissue sections, sectioning artifacts and nuclear overlap result in more complex patterns, which makes the scoring more difficult and time consuming and drives the investigator to a certain bias to only score the fully interpretable nuclei. In addition, the size of the tumor cells and the design of the probe sets may also affect the performance of the test on tissue sections much more than on isolated nuclei. For instance, a larger size of the nuclei and a longer physical distance between two probes obviously will result in more nuclei with an incomplete set of signal because of the cutting artifacts. An alternative approach is to use thick sections (15-20 μm) and to evaluate hybridization signals by confocal microscopy, avoiding sectioning artifacts.[130,131]

***Current view on the diagnosis and classification of Burkitt's lymphoma***

The eponym Burkitt's lymphoma (BL) underscores the unique contribution of Denis Burkitt who described this tumor in children in equatorial Africa in 1961, delineated its geographical distribution and pioneered its treatment.[145] A few years later, a collaborative meeting of the World Health Organization and the International Agency for Research in Cancer defined the characteristic morphology of the tumor and also extended this definition to tumors that occasionally occur in all other parts the world.[146] Currently, three forms are recognized that occur in completely different clinical settings: an endemic form (eBL) found in equatorial Africa and Papua New Guinea, a sporadic form (sBL) arising in non-endemic areas, and human immunodeficiency virus associated BL (HIV-BL).[1, 2, 40,147-150] Although BL comprises only 1-2% of all lymphomas in Western Europe and USA, it represents 30-50% of childhood lymphomas in the same parts of the world, 25-35% of all HIV related lymphomas and it is the most common childhood malignancy in the endemic areas.

From a biological point of view BL is a relatively homogeneous disorder, derived from early centroblasts, present in the dark zone of normal germinal centers.[1,3,40,137] Correspondingly, BL express CD10 and BCL6 and lack BCL2. Most cases carry a reciprocal translocation t(8;14)(q24;q32) or one of its variants resulting in the deregulation of *MYC* gene. [1,38-41] However, BL subtypes show certain differences in their clinical presentation, anatomical distribution of the disease, and association with Epstein-Barr virus (EBV) infection.[1,2,148,149] In addition, both the 8q24 and 14q32 chromosomal breakpoints differ at the molecular level in endemic and sporadic BL patients [41, 87,88- 90], suggesting that different mechanisms and therefore different stages of B-cell development are involved in the generation of chromosomal breakpoints [3,5, 41] (e.g. *IGH* hypermutation and *IGH* class switch recombination, respectively) .

The terms Burkitt's like lymphoma has been introduced into the REAL classification scheme [2] for the cases that lie in this histologic "no man land" between BL and DLBCL, however it caused considerable confusion and misunderstanding in the field.[150,151] In a key manuscript on the reproducibility and clinical impact of the REAL classification, an unacceptable low inter- and intra-observer agreement existed with respect to the histological recognition of Burkitt and Burkitt-like lymphoma.[151]

The recognition and prompt diagnosis of BL versus DLBCL bear important clinical implications, as BL patients benefit from specific chemotherapeutic regimens.[152,153] The recent World Health Organization (WHO) classification took a view that 'atypical Burkitt's/Burkitt's like lymphoma belong to the spectrum of the true Burkitt's, however, using more restricted criteria for its diagnosis: CD10+, BCL6+, BCL2-, Ki-67~100%.[1] Furthermore, due to a certain level of confusion in literature, the term Burkitt's like lymphoma was advised to be omitted. In addition, the presence of the t(8;14) or its variants is highly recommended for the diagnosis of BL and especially its morphological variant aBL .[1,150] However, to the best of our knowledge, the WHO recommendation on the presence of an 8q24/*MYC* breakpoint for the differential diagnosis of BL versus DLBCL has not been validated on a large lymphoma series. While most cases of BL apparently contain an 8q24/*MYC* breakpoint, this aberration is clearly not disease specific as it has been identified in 5-15% of DLBCL, in few FL in high-grade transformation, in MM with a clinically accelerated phase, but also in rare case of lymphoblastic lymphoma/ leukemia and MCL in blastic transformation.[42-48,154]

***The scope of the thesis***

Chapter 1 of this thesis provides general information upon the chromosomal translocations in B- cell lymphomas and their incorporation into current classification scheme. Different approaches for the detection of the chromosomal translocation are briefly reviewed with a special emphasis on the interphase FISH as a preferable detection strategy. Current idea upon the diagnosis and classification of Burkitt's lymphoma is further presented, pointing out possible diagnostic pitfalls and the impact of *MYC* translocation in this field.

In chapters 2 and 3 the detection of the translocation breakpoints by segregation interphase FISH, using routine, fixed, paraffin-embedded tissue sections is described. Both studies used similar tissue pre-treatment protocols; in chapter 2 large multi-YAC's constructs, flanking *MYC* breakpoint region were applied as a probes, while in chapter 3 three small locus specific probe sets, consisting of cosmids and PACs were

tested for the detection of the translocation breakpoints within BLC1, *BCL2* and *MYC* breakpoint regions. With all probes we achieved consistent, sufficient FISH signals using various patients' samples. Different algorithms for evaluation and interpretation of FISH signals on routine paraffin sections were tested and compared.

Detection of the *MYC* translocation breakpoints by interphase segregation FISH is the subject of chapter 4. Two probes sets, flanking the breakpoint region of more than 1Mb were tested on 40 cell lines and lymphoma samples with 8q24 abnormalities and applied on a series of Burkitt's lymphoma from three different geographic regions. The presence of *MYC* translocation breakpoint is identified in conjunction to the morphology, immunophenotype, EBV association and geographical origin of the samples. Further analysis of the lymphoma samples that lie into the histological "no man's land" between adult BL and DLBCL is presented in chapter 5. Our data indicate that a subset of DLBCL share not only similar (immuno-) morphology with BL but is also relatively frequently carry *MYC* translocation breakpoints.

Four cases of non-endemic, EBV positive BL, associated with unusual florid granulomatous reaction are presented in chapter 6.

Chapter 7 review the data on association of different Ig translocation with MM and related plasma cell disorders, their impact on the biology of disease and patient's outcome.

In chapter 8 we report on FISH analysis of the 11q13 breakpoint and the chromosome 11 numerical status in conjunction to Cyclin D1 RNA and protein expression in 46 MM samples. In addition in the same MM series we evaluate the concurrent expression of another 11q13 oncogene *MYEOV*.

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## Chapter 2

# *MYC* translocations in "Burkitt-like" lymphoma: a FISH study of paraffin embedded biopsy samples

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The detection of chromosomal translocations by fluorescent *in situ* hybridisation (FISH) is widely performed, but very few studies have attempted to apply this technique to paraffin embedded routine biopsy samples. Here we report a study in which paraffin sections from 36 B cell lymphoma biopsies were analysed for the presence of *MYC* translocation breakpoints by means of FISH. The probes consisted of multi-YAC constructs that flanked the breakpoint region therefore separate upon a chromosomal translocation and generate split signals (rather than a more ambiguous "co-localisation" pattern obtained when the two partners in a hybrid gene are detected). Results were assessed by a simple approach that does not require counting of signal numbers per nucleus and is hence more appropriate for use in routine practice. A total of 19 of the 36 lymphomas were scored as positive for *MYC* translocation, and this included 16 of the 20 cases in which classical cytogenetics had shown the presence of the (8;14) translocation (or one of its two variants). We conclude that this two-colour "split signal technique" based on breakpoint flanking probes can be readily applied to detect chromosomal translocation in paraffin embedded tissue sections. Furthermore, our results suggest that the controversial category "Burkitt-like" lymphoma is a heterogeneous disease with respect to translocations involving the *MYC* oncogene, immunophenotype and clinical features, at least in adult patients.

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## Introduction

In recent years non-random cytogenetic abnormalities (e.g. chromosomal translocations) have been identified in a wide variety of haematological malignancies and solid tumours, and they frequently contribute directly to neoplastic transformation (by activating a quiescent oncogene or by creating an oncogenic fusion protein). This direct causal role in the malignant process may account for the fact that these genetic lesions are often more specific as markers of different tumour categories than are morphological features or immunophenotypic profiles.

Unfortunately cytogenetic analysis requires fresh tissue samples and is a labour intensive technique with a relatively low success rate, so that it is not readily applied in the setting of a routine pathology laboratory. A simpler alternative is to use fluorescence *in situ* hybridisation (FISH) on interphase nuclei to detect both numerical and known structural aberrations. However, this technique is also conventionally performed using fresh or frozen samples, limiting its suitability for the routine diagnostic laboratory.

For this reason, attempts have been made to perform FISH on routine paraffin embedded tissue sections, but they have been hampered by technical problems (e.g. high background, weak or inconsistent hybridisation signals, ambiguities in interpreting the results), and relatively low success rates have been reported (Braziel *et al*, 2001; Nolte *et al*, 1996; Nolte *et al*, 1995).

In this paper we report FISH analysis on a series of routine human lymphoma biopsies using large multi-YAC probe constructs that flank the *MYC* region on chromosome 8 where translocation breakpoints occur in Burkitt's lymphoma. These included lymphoma samples that had been studied previously by classical cytogenetic techniques, allowing validation of the FISH results.

We also analysed 26 lymphoma cases of the controversial "Burkitt-like" category for the presence of MYC translocation, and correlated the results with immunophenotypic and clinical data.

In many *in situ* hybridisation protocols the final evaluation involves extensive counting procedures to identify different signal patterns, followed by statistical analysis. However, in this study we adopted a non-numerical approach to reading the results, similar to the "eyeballing" assessment that pathologists make of immunohistochemically labelled sections in routine practice.

## Materials and Methods

*Tissue samples:*

Routinely processed formalin or Bouin's fixed paraffin embedded lymphoma samples were obtained from the authors' institutions. A total of 20 lymphoma samples (of different morphological subtypes) were selected on the basis that the (8;14) translocation (or a variant) had been previously detected by conventional cytogenetic analysis (see table1A).

In addition, 16 samples of the controversial "Burkitt-like" category (BLL) were studied. (Table 1B). These cases shared some of the features of Burkitt's lymphoma (BL), e.g. high proliferative pattern, "starry sky" appearance, cohesive growth pattern however differed in their cytomorphology and/or immunophenotypic prifile.

*Immunophenotyping*

Immunohistochemical labelling of paraffin sections was performed for the following markers: CD20 (antibody L26), Ki-67 (antibody MIB1), bcl-2 (antibody 124) and CD10 (antibody 56C6). The first three antibodies were obtained from DAKO, the last one from Novacastra, Newcastle, UK.

*Cytogenetic studies*

These were performed by conventional methods using G- or R- binding techniques on cells isolated from fresh tissue biopsies.

*Fluorescence in situ hybridisation*

*MYC probes:* The probe sets consisted of two multi-YAC constructs flanking the *MYC* translocation breakpoint region of 1Mb. Their generation and detailed characterisation was described previously (Davison *et al*, 1998). Probes were labelled with either FITC or biotin by random priming (Invitrogen, Paisley, UK).

*In situ hybridisation of paraffin sections:* Dewaxed 4 μm paraffin sections were pressure cooked for 8 minutes in a microwave oven in Tris/EDTA buffer solution (50 mM Tris base/2 mM EDTA, pH9.0) to make target DNA accessible to the probes. Additional tissue pre-treatment included RNAase (Sigma, Poole, UK) 100μm/ml/ 2 X standard citrate saline (SSC) pH 7.0 for 1 hour at 37°C, followed by 30 minutes incubation with pepsin (Sigma, Poole, UK), 50 μg/ml in 0.1 mM HCL at 37°C. To end the reaction, the slides were placed in a stop buffer (50 mM $MgCl_2$/PBS) for 5 minutes and washed 2 X 5 minutes in PBS (pH 7.4).Denaturing was performed in 70% formamide (Sigma, Poole, UK) /2 X SSC for 15 minutes at 80°C.The

*MYC* probes, diluted to 100-200 ng/µl in hybridisation mix (50% formamide/10% dextran sulphate / 2 x SSC) were denatured at 80°C for 5 minutes and an aliquot (10 µl-20 µl) was then placed onto denatured slides. Hybridisation was performed in a humidified chamber overnight at 37°C.

*Post-hybridization washes and detection:* After removing the coverslips, the slides were washed 2 X 5 minutes in 0.4 X SSC/0.05% Tween 20 at 72°C, followed by 2 X 5 minute washes in 2 x SSC/0.05% Tween 20 and a final 5 minute wash in 4 X SSC /0.05% Tween 20. To avoid non-specific labelling, the slides were incubated in blocking buffer (5% non-fat milk, 2 X SSC /0.05% Tween 20) at 37°C for 10 minutes. Biotinylated probes were detected with Texas Red-Avidin DSC, biotinylated goat-anti-Avidin, and Texas Red-Avidin DSC (Vector Laboratories, Burlingame, CA, USA). The signals of FITC-labelled probes were amplified by mouse anti-FITC and FITC-conjugated goat anti-mouse Ig (DAKO). All the reagents were diluted in blocking buffer according to the manufacturer's instruction. The incubations were performed for 30 minutes at 37°C with intervening 3 X 5 minute washes in 4 X SSC/0.05% Tween 20. After counterstaining with DAPI the sections were mounted in DAKO fluorescence maunting medium (DAKO).

*Fluorescence microscopy*

The samples were evaluated on a Zeiss fluorescent microscope, equipped with Texas Red, FITC, DAPI and triple filters under 100 X oil objective. Images were recorded as previously described using Labvision and Photoshop software (Improvision and Adobe respectively) running on an Apple Macintosh computer (Cheong *et al*, 1995). The samples were evaluated independently, without knowledge of the cytogenetic results.

**Results**

Examples of the patterns observed are shown in Fig. 1. Areas that were suitable for assessment (e.g. in which tissue structure was intact) were evaluated, ignoring areas of poor hybridisation or over-digestion and also areas where the tissue is too dense to give interpretable results.

A simple approach to assessment of FISH results summarised in Fig. 2 was established. This scoring procedure eliminates from consideration cells that are uninterpretable because of artefactual phenomena (e.g. partial loss of the nucleus during sectioning, excessive non-specific signals) and lends itself to use in a routine diagnostic setting.Translocations involving the *MYC* gene were identified by this non-numerical approach when a specific signal pattern (by co-localisation of one pair of signals and segregation of the other) was seen. The results from FISH, together with immunohistochemical and clinical data, are summarised in Table 1. Cells with a FISH pattern indicative of *MYC* translocation (Figs. 2 and 8) were detected in 16 out of the 20 cases in which the (8;14) translocation or one of its two variants had been detected previously by cytogenetics (see Figs. 1c and d). Among the 16 cases selected on the basis of "Burkitt-like" morphology, three samples showed evidence of translocation. In addition, one case showed amplification of the intact *MYC* gene, indicated by the presence of multiple co-localised signals (Fig 1e).

Twenty six lymphoma samples in this series that shared "Burkitt-like" features were reviewed together in relation to the presence or absence of a translocation involving *MYC* (as detected by classical cytogenetics, and/ or by FISH) (see table 1A and B). All but one patient were adults and in half of them translocations of *MYC* gene have been identified. The *MYC* breakpoint positive patients were slightly younger (age 12-80, median 40) than *MYC* breakpoint negative patients (34-90, median 58) with tendency of male prevalence (9 out of 13, *MYC* breakpoint positive, and 7 out 13, *MYC* negative) and extranodal site involvement (6 out of 13, translocation positive, and 3 out of 13 translocation negative).

Twenty three of "Burkitt-like" lymphoma samples were immunostained for CD10, which was expressed in 9 out of 12 translocation positive cases and in 6 out of 11 translocation negative cases. The staining pattern for BCL2 showed the opposite tendency as 10 out of 13 translocation negative samples, expressed BCL2 in contrast to 5 out of 13 translocation negative samples.

Six patients co-expressed CD10 and BCL2 and 1 lacked both markers.

**Discussion**

In recent years fluorescence *in situ* hybridisation has been used extensively to study genetic alterations in interphase cell preparations from smears, cell suspensions of cells isolated from fresh or frozen tissue samples. However most of the studies using FISH on routine processed paraffin embedded tissue have been restricted to the detection of numerical aberration (e.g. trisomy, chromosome loss), and there have been few reports of the detection of chromosome translocations in routine biopsy samples of tumour tissue (Biegel et al,995; Nolte et al, 1995, 1996; Davison et al, 1998; Zilmer et al, 1998; Birdsall et al,1999; Cataldo et al, 1999; Kumar et al, 1999; Li et al, 1999; Lu et al, 1999; Remstein et al, 2000; Braziel et al, 2001).

**Table 1. Details of lymphoma cases, investigated in the present study**
**A. Cases, previously studied by cytogenetics**

| Case no. | Sex | Age | Site | Histology | Ki67 | CD10 | BCL2 | FISH | Karyotype |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 23 | Liver | Burkitt's | 80% | Pos | Neg | Pos | t(8;14) |
| 2 | M | 32 | Subcutaneous nodule | Burkitt's | 90% | Pos | Neg | Pos | t(8;22) |
| 3 | M | 77 | Lymph node | Burkitt's | 90% | Pos | Neg | Pos | t(8;14) |
| 4 | M | 17 | Stomach | Burkitt-like | 95% | Pos | Neg | Pos | t(8;14) |
| 5 | M | 24 | Lymph node | Burkitt-like | 95% | Pos | Neg | Pos | t(8;14) |
| 6 | M | 35 | Stomach | Burkitt-like | 95% | Pos | Neg | Pos | t(8;14) |
| 7 | M | 34 | Stomach | Burkitt-like | 95% | Pos | Neg | Pos | t(8;14) |
| 8 | F | 70 | Lymph node | Burkitt-like | 90% | Neg | Pos | Pos | t(8;14) |
| 9 | M | 41 | Thoracic wall | Burkitt-like | 90% | Pos | Neg | Pos | t(8;14) |
| 10 | M | 80 | Lymph node | Burkitt-like | 80% | Pos | Pos | Pos | t(8;14) |
| 11 | F | 56 | Lymph node | Burkitt-like | 90% | Neg | Pos | Neg | t8;14) |
| 12 | M | 56 | Lymph node | Burkitt-like | 80% | NA | Pos | Pos | t(8;14) |
| 13 | M | 61 | Lymph node | DLBCL | 80% | Pos | Pos | Pos | t(2;8) |
| 14 | M | 65 | Retroperitoneal tumour | DLBCL | 90% | NA | Pos | Pos | t(8;22) |
| 15 | M | 57 | Lymph node | DLBCL | 50% | Neg | Pos | Pos | t(8;14) |
| 16 | F | 47 | Lymph node | FL grade III | 90% | Pos | Neg | Pos | t(8;14) |
| 17 | F | 72 | Lymph node | MCL, blastoid variant | <50% | Neg | Pos | Pos | t(8;22) |
| 18 | M | 42 | Lymph node | MCL, blastoid variant | <50% | Neg | Pos | Neg | t(8;14) |
| 19 | F | 63 | Lymph node | Burkitt-like | <50% | NA | Pos | Neg | t(2;8) and t(8;14) |
| 20 | M | 66 | Lymph node | DLBCL | <50% | Pos | Pos | Neg | t(8;22) |

**B. Large B cell lymphomas (not studied by cytogenetics) that showed "Burkitt-like" or "atypical Burkitt's" features**

| Case no. | Sex | Age | Site | Ki67 | CD10 | BCL2 | FISH |
|---|---|---|---|---|---|---|---|
| 21 | F | 21 | Large intestine | 95% | Pos | Neg | Pos |
| 22 | F | 42 | Stomach | 95% | Pos | Neg | Neg |
| 23 | M | 64 | Lymph node | 50% | NA | Pos | Neg * |
| 24 | F | 67 | Ovary | 90% | NA | Pos | Neg |
| 25 | F | 34 | Small intestine | 95% | Pos | Neg | Neg |
| 26 | F | 91 | Lymph node | 80% | Neg | Pos | Neg |
| 27 | M | 49 | Lymph node | 80% | Neg | Pos | Neg |
| 28 | F | 79 | Lymph node | 90% | Pos | Pos | Neg |
| 29 | M | NA | Lymph node | 90% | Neg | Pos | Neg |
| 30 | F | NA | Lymph node | 99% | Pos | Pos | Neg |
| 31 | M | 62 | Lymph node | 85% | Pos | Pos | Neg |
| 32 | M | 34 | Lymph node | 95% | Pos | Neg | Neg |
| 33 | M | 55 | Lymph node | 95% | Neg | Pos | Neg |
| 34 | M | 23 | Lymph node | 95% | Pos | Neg | Pos |
| 35 | M | 12 | Tonsil | 95% | Neg | Neg | Pos |
| 36 | M | 62 | Lymph node | 95% | Neg | Pos | Neg |

* - amplification of *MYC* gene; DLBCL - Diffuse large B-cell lymphoma; FL - Follicular lymphoma; MCL- Mantle cell lymphoma; NA - Data not available.

In the present study we report the use of this approach to detect translocations involving the MYC gene in routine lymphoma tissue sections, obtained from several different laboratories. The technique we used differed in several respects from previous reports. Firstly, several FISH studies have been performed on isolated nuclei obtained by dissociation from paraffin sections (Colleoni *et al*, 2000; Kumar *et al*, 1999; Li *et al*, 1999; Poetsch *et al*, 1999; Remstein *et al*, 2000). However the application of FISH on routine paraffin sections allow to study the translocations in their histological context, needs less patients material and is more close to the routine pathology settings. To avoid problems of truncation of the nuclei some studies (Aubele *et al*, 1997; D'Alessandro *et al*, 1997) report on the use of thick (i.e. 15 - 20µm) sections. Of note, this approach hampers hybridisation efficiency in addition and needs special equipment (confocal microscope) for evaluation of the results, which would make again the technique of little use in a routine context.

In the present study we evaluated and analysed the results on conventional computer-based fluorescence microscopy system, which has already been used extensively in one of the authors' laboratories for evaluating and recording immunofluorescent labelling of paraffin embedded tissue sections (Mason *et al*, 2000).

A scoring system such as the one proposed in this study (see Fig 2) should eliminate from consideration nuclei that are truncated or in which hybridisation has been sub-optimal. Evaluation of *in situ* hybridisation frequently involves counting the number of signals associated with a specified number of cells (including negative controls to establish threshold values) but in the present study we made a subjective assessment of the presence of a translocation involving the *MYC* gene, comparable to the non-numerical evaluation of immunohistological labelling with which pathologists are familiar. As a result of our experience we recommend the strategy summarised in Fig. 2, since *in situ* hybridisation is unlikely to be adopted in routine diagnostic practice if its results cannot be rapidly assessed.

Finally in our study we used breakpoint flanking probes in FISH segregation assay instead of more traditional usage of probes that hybridise to the two different translocation partners in the hybrid gene (e.g. *PML–RARA* and *BCR-ABL*). This technique, in which co-localisation of the two probes indicates the presence of a translocation, has been widely used (Biegel *et al*, 1995; Birdsall *et al*, 1999; Colleoni*et al*, 2000; Kumar*et al*, 1999; Li*et al*, 1999; Lu *et al*, 1999; Nolte*et al*, 1995; Taniwaki *et al*, 1995; Zilmer *et al*, 1998). However it suffers from relatively high rate of false positivity due to random co-localisation of the signals in the interphase nuclei (Chase *et al*, 1997), and is likely to rise an even higher level of false positivity on tissue sections because of the overlapping of the nuclei.

Of note, many genes involved in translocations (*MYC, BCL6, ALK)* have more than one translocations partners which is again in favour of the use of FISH segregation approach for regular detection of translocation breakpoints.

More complex FISH approaches (e.g. additional probe labelled in a third colour and others) give rise to complicated and uninterpretable FISH results in tissue sections and do not seem appropriate for routine use.

However in this study we used breakpoint flanking probes that produced bright contrasting signals on paraffin sections and are unlikely to generate false positive results. This FISH segregation approach has been used in studies of a number of genes involved in translocations, e.g. *ALK* (Cataldo *et al*, 1999), BCL-1 (Coignet *et al*, 1996), *MYC* (Davison *et al*, 1998), *EWS* (Davison *et al*, 1998) and *BCL-2* (Vaandrager *et al*, 2000)) but its application on routine paraffin sections remained limited.

The results we obtained in the present study suggest that technical obstacles related to the application of FISH technique on paraffin tissue could be avoided by using appropriate tissue pretreatment protocol.

We analysed by means of FISH 36 routinely processed lymphoma samples for presence of *MYC* translocation breakpoint. However in 4 samples with previously proven translocation by conventional cytogenetics we fail to detect it by means of FISH. These samples are under further investigations. It is very unlikely *MYC* translocation breakpoint to be located out of the range of the probes as they flank a genomic region of more than 1Mb 5' and 3' of the gene. It is possible the translocation to be confined to a small proportion of the tumour cells and to be missed in the process of evaluation of he samples.

It is of interest that most of the unselected cases categorised as "Burkitt-like" lymphoma for which no cytogenetic data were available lacked evidence of *MYC* translocation. However, *MYC* breakpoint can clearly occur in such cases, since ten of the 20 lymphomas studied because they were known to carry an (8;14) translocation (or one of its variants) were classified as "Burkitt-like" tumours. The REAL classification took the view that lymphomas with "Burkitt-like" morphology probably belong to the spectrum of diffuse large cell lymphoma (Harris *et al*, 1994). However the category was not reproducible even between experienced haematopathologists (<50% agreement in the International Non-Hodgkin's Lymphoma Study). Moreover clinical data indicated that at least in children tumours classified as BLL behave as true BL. The recently published WHO classification implies more stringent criteria (Ki67 labelling index ~100%) and categorises "atypical Burkitt/Burkitt-like" lymphoma with true Burkitt's lymphoma (adding however that this term should be "reserved for cases with proven or strong presumptive evidence of *MYC* translocation").Our data on cases with "Burkitt-like" features, and data from Brazial et al (Braziel *et al*, 2001) who observed *MYC*

translocation in four of five such cases, indicate that some cases carry a breakpoint involving *MYC* but that others may lack this anomaly. Our results also suggest, in keeping with a previous study of Braziel et al (Braziel*et al*, 2001), that expression of CD10 and absence of BCL-2 are features that tend to pick out some but not all of translocation-positive "Burkitt-like" neoplasm. However the patients in our series are clearly heterogeneous in respect to available clinical data, antigen expression profile and *MYC* translocation and seems not to represents disease entity. In addition high proliferative index shared by the tumours in our series could possibly be related to other underlying genetic lesions in cases lacking *MYC* translocation. Of note, *MYC* translocation is also not strictly disease specific and could occur in different disease entities (see table 1A). As the group included mainly adult patients it would also clearly be of interest to analyse larger series including also young patients and to correlate the presence or absence of *MYC* breakpoint with clinical outcome.

In conclusion, our study suggests that FISH analysis on paraffin embedded sections in technically feasible and could be performed in routine pathology settings both for diagnostic and research.

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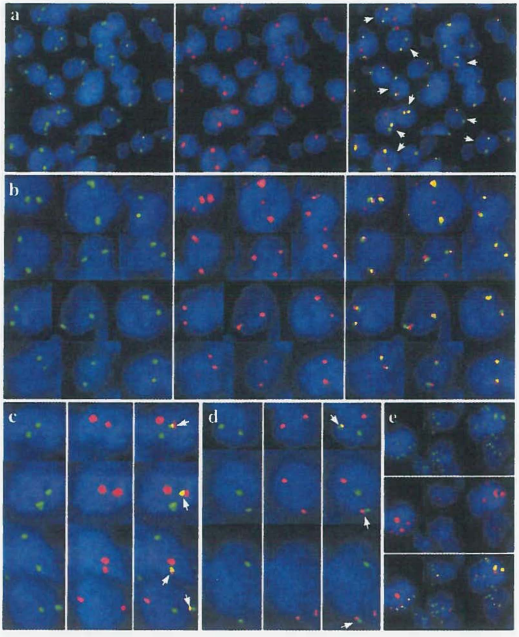


**Figure 1.** Dual colour FISH segregation assay on routine paraffin embedded lymphoma biopsies using two MYC breakpoint flanking probes. Each part of the image (a - e) shows hybridisation signals of an area with the FITC-labelled probe (hybridising telomeric to the breakpoint region,) with the Texas Red-labelled probe (hybridising centromeric to the breakpoint region), and with both probes simultaneously. All images also show the DAPI nuclear counterstain. (a) Low power of a large B cell lymphoma. Many cells show co-localised red and green signals, indicating normal cells (representative examples arrowed). (b) A B cell lymphoma of "Burkitt like" morphology at higher power showing twelve cells, all with coincident signals, indicating that this tumour, despite its morphology, probably does not carry one of the classical Burkitt's-associated translocations. (c and d) Two lymphomas showing a pair of segregated and a pair of co-localised (arrowed), signals indicating mono-allelic translocation breakpoint involving the MYC gene. In one of these cases, cytogenetic analysis had been performed on fresh tissue and had shown the presence of an (8;14) translocation. (e) A large B cell lymphoma, showing amplification of MYC, as indicated by multiple co-localised signals.

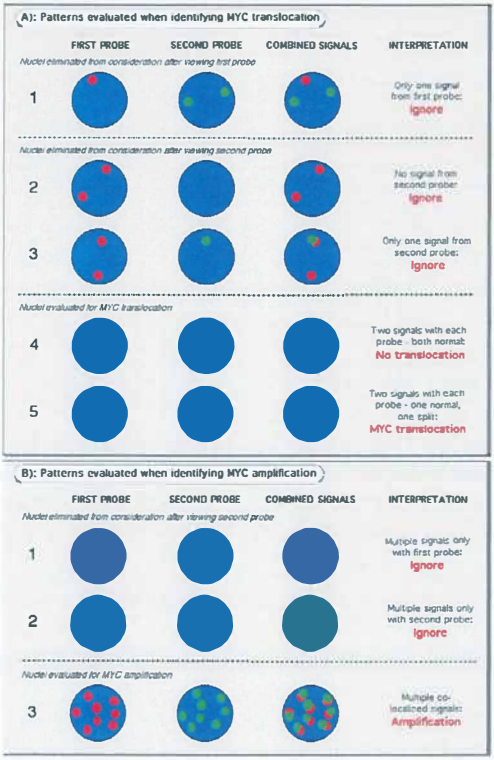


**Figure 2.** Strategy for evaluating FISH results for translocation of the MYC gene in paraffin embedded tissue biopsies (and approach that should be equally applicable to other genes involved in translocations).
Step 1: The section is scanned for a nuclei which contain two specific, hybridisation signals with one of the probes (preferably the probe that gives the least non-specific background reactivity). This step selects nuclei that were likely to contain both MYC alleles and should eliminate from further consideration nuclei in which one of the signals has been lost during tissue sectioning, or hybridisation was not efficient. Nuclei which show only one signal or multiple signals (1 and 2 respectively) are not considered further. Exception are the nuclei showing multiple specific signals for both probes were, indicating the presence of amplification.(3)
Step 2: The fluorescent filter set is then changed to view the signals from the other probe on any nuclei fulfilling these criteria, but the result is ignored if there is no signal (4), only one signal (5) or multiple signals (6) (indicating excessive non-specific hybridisation).
Step 3: The nucleus can then be assigned to one of two categories, representing the absence or presence of mono-allelic MYC translocation breakpoint (7 and 8 respectively).

## Chapter 3

# Detection of three common translocation breakpoints in non-Hodgkin's lymphomas by fluorescence *in situ* hybridization on routine paraffin-embedded tissue sections

Eugenia Haralambieva, Karin Kleiverda, David Y Mason, Ed Schuuring, Philip M Kluin

Non-random chromosomal translocations are specifically involved in the pathogenesis of many non-Hodgkin's lymphomas and have clinical implications as diagnostic and/or prognostic markers. Their detection is often impaired by technical problems, including the distribution of the breakpoints over large genomic areas. This study reports a fluorescence *in situ* hybridization (FISH) method which allows the detection of specific chromosomal breakpoints in tissue sections from routinely fixed, paraffin-embedded samples. Hybridization was performed after demasking the DNA. Previously validated locus-specific probes (cosmids, PACs) flanking the BCL1, *BCL2* regions and similar new probes for the *MYC* breakpoint region were used. The cases studied were five mantle cell lymphomas (MCL) and five follicular lymphomas (FL), selected on the basis of a previously proved *t*(11;14) and *t*(14;18) and five randomly chosen Burkitt's lymphomas (BL), as well as 21 negative control samples. In all samples, hybridization signals of sufficient intensity were obtained. Three different algorithms were used to score the hybridization signals in tissue sections, two of them taking into account the nuclei and their signal distribution indicative of chromosomal break, and one only considering the colocalization or segregation of the signals.

In control tissues, these algorithms resulted in cut-off levels of 9.1%, 1.3%, or 10.0%. In the 15 lymphoma samples the percentages of abnormal cells/signals ranged from 28% to 80%, 13% to 49%, and 40% to 70%, respectively. The results indicate that small locus-specific probes can be used in FISH for regular detection of translocation breakpoints on routine paraffin tissue sections.

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## Introduction

During the past decade the association of distinct types of non-Hodgkin's lymphomas (NHL) with certain recurrent genetic abnormalities has been extensively studied. Many of these abnormalities include chromosomal translocations which are involved in the pathogenesis of the disease, through mechanisms including activation of a proto-oncogene via transcriptional deregulation such as BCL1/*CCND1*, *BCL2*, *BCL6*, *MYC* [1,2] or the generation of a fusion gene such as *NPM-ALK* [3]. Apart from their pathogenic relevance, many genetic lesions represent molecular markers of the disease and have diagnostic and clinical implications [4]. Conventional cytogenetic analysis is feasible in only a few cases, as it requires fresh tissue samples and dividing cells. On the other hand, the lack of local clustering of the breakpoints (e.g. BCL1/*CCND1* and *MYC*) and heterogeneity of the translocation partners (e.g. *BCL6*) make PCR and Southern blot less applicable for regular diagnostics [5,6]. In some instances, an abnormally expressed protein product, such as cyclin D1 in MCL, strongly indicates the presence of a translocation breakpoint and can be detected by immunohistochemistry. However, no such correlation exists in many other cases, such as BCL2 and BCL6 in diffuse large B cell lymphoma. Breakpoint analysis, therefore, cannot simply be replaced by immunohistochemistry for the respective oncogene products. Fluorescence *in situ* hybridization (FISH) is a relatively simple technique that allows the detection of both numerical and defined structural chromosomal abnormalities and also provides reliable information at the single-cell level [5,7]. It has been extensively applied on metaphase spreads or intact Interphase nuclei obtained from imprints, cell suspensions, or frozen tissue. However, its application on routine paraffin-embedded tissue sections is impaired by technical problems, such as weak and/or inconsistent hybridization signals, low signal-to-noise ratio, and ambiguities in interpreting the results [8–14].

Here we report on the successful FISH analysis of three common chromosomal translocations on routine paraffin-embedded tissue sections. BCL1, *BCL2* (3' of the mbr/mcr region) and *MYC* translocation breakpoints were detected using small locus-specific probes including single PACs and cosmids. The probe sets for BCL1 and *BCL2* have already been extensively tested on nuclear suspensions from fresh or frozen tissue samples [15–17]. A full contig of *MYC* probes was mapped using DNA fibre FISH and an appropriate probe set was selected on the basis of the available literature on *MYC* breakpoints in non-endemic BL [18–20]. Together with an additional probe set covering the far 5'region of *MYC* involved in endemic BL [18–21], it will be the subject of a more detailed study on BL (Haralambieva *et al*., in preparation).

Two-colour FISH breakpoint analysis can be performed using a colocalization assay with probes that are specific for the two different loci and that are randomly localized in normal nuclei, but colocalize upon chromosomal breakage and juxtaposition of the two loci; or a segregation assay in which two breakpoint flanking signals colocalize in normal nuclei, but segregate upon chromosomal breakage. Combinations of both assays are also possible. Segregation assays have a number of advantages over colocalization assays. Firstly, due to the fortuitous overlap of signals in normal cells, the cut-off levels are generally much higher in FISH colocalization assays than in segregation assays [22]. Secondly, some authors described a non-random distribution in nuclei especially for the genes/loci that are involved in translocations, further increasing the chance of colocalization in normal cells [23–25]. Thirdly, some target genes such as *MYC* and *ALK* have variable partners to which they are juxtaposed and, in the case of a colocalization assay, probes for all partners should be used. Finally, in tissue sections, both cutting artefacts and the overlap of nuclei generate loss of signals, as well as additional fortuitous overlap. In a colocalization assay, these patterns are difficult to recognize and interpret. In contrast, using appropriate nearby probes in a segregation assay, each normal cell should be easily recognizable by the presence of one or two sets of overlapping signals and each single-coloured signal should indicate a chromosomal breakage. In the present study, a maximal distance of approximately 800 kb was chosen to avoid false segregation of signals in normal Interphase nuclei.

Several algorithms for the interpretation of the FISH results were tested, two of these taking into account the number and distribution of signals in each individual nucleus, and one considering only the presence of segregated versus colocalized signals, irrespective of their nuclear distribution.

## Materials and methods

### *Tissue samples*

Routinely processed paraffin-embedded biopsy samples of five patients with MCL, five patients with FL, and five non-endemic BL patients were retrieved from the files of the Department of Pathology, Leiden University Medical Centre, and the Department of Pathology, University Hospital Groningen, The Netherlands. The samples dated from 1986 to 2000 and were fixed in 10% phosphate-buffered formalin solution pH 7,0 and routinely processed in multiple pathology laboratories. All MCL and FL samples had characteristic morphological and immunophenotypic features [4,26] and were selected on the basis of previously demonstrated BCL1/*IgH* and *BCL2*/*IgH* translocations, respectively [15–17]. All BL patients were children or young adults with an abdominal tumour, showing unambiguous morphological and

immunophenotypic characteristics of BL [4,26]. For controls we applied every probe set on paraffin sections of three hyperplastic tonsils and on sections of four other lymphomas in which the specific breakpoint was not expected; for instance, two MCL and two BL were used as negative tumour controls for the *BCL2* probe set.

***Probe sets***

Originally, the two probe sets for BCL1 (5' of *CCND1* gene) and *BCL2* (mbr/mcr region) were designed using a combination of DNA fibre FISH and Interphase FISH [15–17]. In brief, for mapping of the probes and subsequent mapping of all possible breakpoints, DNA fibre FISH was used on normal and lymphoma DNA fibres, respectively. Based on these data, optimal probe sets for interphase FISH were selected (Figure 1, BCL1 and *BCL2*). Subsequently, these sets have been extensively tested on large series of clinical material. For detection of the breakpoints in the immediate 5' region and the intron I region of *MYC*, as reported in most sporadic Burkitt's lymphomas [18–20], we selected two pooled cosmids (cos I C8 and cos II F5) and PAC 1117K22 (Figure 1, *MYC*) which, according to the published data, should flank the breakpoint region in most nonendemic BL.

All probes were labelled by standard nick translation with biotin-16-aUTP or digoxygenin-11-dUTP (Roche, Basel, Switzerland) and dissolved in the hybridization solution consisting of 50% formamide, 10% dextran sulphate, 50 mM sodium phosphate, and 2X standard saline citrate (SSC) pH 7.0.

***Sample preparation***

Approximately 4 µm thick tissue sections were placed on aminopropyltriethoxysilane (APES, Sigma-Aldrich, Chemie, Steinheim, Germany) coated slides, dewaxed and rehydrated. The target demasking protocol consisted of 10 min pressure cooking in 50 mM Tris/2 mM EDTA buffer solution, pH 9.0, after which the slides were washed in 2X SSC. RNase treatment using 100 µg/ml RNase (Roche, Basel, Switzerland) was performed at 37°C for 1 h followed by enzymatic digestion with 0.1% pepsin (Sigma-Aldrich) pH 2.0 at 37°C for 10 min. The tissue samples were then washed with phosphate-buffered saline (PBS) pH 7.4 and dehydrated in 70%, 90%, and 100% ethanol. Denaturing was performed on a slidewarmer at 80°C for 12 min in a solution containing 70% formamide, 50 mM sodium phosphate, 2X SSC, pH 7.0, under a coverslip. Slides were washed in ice-cold 2X SSC for 2 min, dehydrated in 70% (–20°C), 90%, and 100% ethanol and then airdried.

***Hybridization and detection***

Aliquots of labelled DNA of each probe were diluted to a final concentration of 3 ng/µl in 50-fold excess Human Cot 1 DNA (Invitrogen Life Technologies, Paisley, UK) in hybridization solution, denatured at 80°C for 8 min and annealed at 37°C for 30 min. Probe mixtures, 10–20 µl per section, were placed onto denatured slides and covered with glass coverslips. The hybridization was carried out in a humidified chamber at 37°C overnight. Excess of the probe was washed in 2X SSC/0.1%Tween (3 X5 min at 37°C), 0.1X SSC (3 X 5 min at 60°C) followed by Tris/NaCl/0.05% Tween, pH 7.5 for 5 min at room temperature. Immunodetection was performed as previously described [27].

***Evaluation***

Slides were analysed with a Leica DMRXA fluorescence microscope equipped with PL Fluotar 10x, 40x and 100x objectives and triple band-pass filter for FITC, Texas Red and DAPI (Leica). For documentation, images were captured using a COHU 4910 series monochrome CCD camera (COHU, San Diego, CA) attached to the fluorescence microscope and Leica QFISH software (Leica Imaging Systems, Cambridge, UK). Images were processed with Adobe Photoshop (version 4.0; Adobe Systems, Mountain View, CA).

Hybridization signals were enumerated in 200 morphologically intact nuclei for each sample. To avoid detection of signals in overlapping nuclei, an inherent problem in tissue sections, nuclei were counted mainly at the periphery of the section and/or at the places where a lower density enabled recognition of individual cells. Alternatively, 200 hybridization signals (single against colocalized) were counted in tissue sections without looking at the individual nuclei. Signals were considered colocalized when the distance between them was equal to or smaller than the size of one hybridization signal.

Statistical analysis of the results was performed using the Mann–Whitney test for comparison of two independent groups.

**Results**

***Hybridization efficiency and analysis in negative controls:*** For all probes, hybridization signals of sufficient intensity were obtained (Figure 2). No cases were excluded because of a weak signal. Of note, we observed no significant variation in hybridization efficiency related to the date and origin of the sample. Furthermore, the DNA demasking protocol allowed us to use a fixed pepsin concentration and digestion time.

Although in a pilot study a readable hybridization signal was obtained using probe sets consisting of two single cosmids, we used a single PAC and/or two pooled cosmids to obtain a comparable intensity of signals (Figures 1 and 2). As seen in Figure 1, the three probe sets had a separation of 250 kb for the *BCL2* mbr/mcr breakpoints, 400 kb for the *MYC* breakpoints, and 800 kb for BCL1 breakpoints. In the 21 negative controls (for each breakpoint three tonsils and four lymphomas without a breakpoint) hybridized with these probe sets, segregated signals (see Figure 3, patterns C–H) were found in 1–7% of the nuclei (mean 4.0%, standard deviation 1.7%). Therefore, the cut-off level was set up at 9.1% (mean plus three times standard deviation; see also Figure 4, BCL1, *BCL2*, and *MYC*). There were minor differences between the three probe sets: with the BCL1 probe set, single segregated signals were detected in 1–7% (mean 4.9%, standard deviation 1.9%) of the nuclei, with the *BCL2* probe set in 1–4% (mean 3.0%, standard deviation 1.2%), and with the *MYC* probe set in 1–5% (mean 4.2%, standard deviation 1.5%) of the cells. These differences paralleled the different distances between the flanking probes for each probe set (Figure 1). Nuclei with two colocalized and two segregated signals (Figure 3, pattern C), recognized as the most stringent indication for a mono-allelic breakpoint, were exceedingly rare (0–1%, mean 0.15%, standard deviation 0.37%; see also Figure 4, pattern C) in negative control tissue samples. A third option for evaluation of the FISH results is to score only the type of signal (colocalized against separated) without looking at individual cells and assuming that the presence of any single signal indicates a true breakpoint. In negative control tissues, 1–9% (mean 3.7%, standard deviation 2.1%) single signals were counted and the threshold level was set at 10.1%.

One follicular lymphoma, which initially was used as a negative control for assessment of *MYC* breakpoints, showed segregated *MYC* signals in 43% of the nuclei and segregated *BCL2* signals in 68% of the nuclei. This case probably represented an FL with bi-allelic *BCL2* and *MYC* breakpoints [28] and was therefore excluded from the control group for *MYC*.

***Detection of BCL1, BCL2 and MYC breakpoints in lymphomas:*** Breakage within the BCL1, *BCL2*, and *MYC* breakpoint region should result in segregation of one red and one green signal. However, due to the cutting artefacts and the admixture of normal cells, this will result in at least eight different signal patterns (Figure 3, patterns A–H, Figure 2B and C). In aneuploid tumours, even more patterns may be envisaged.

The scoring results for five FL, BL, and MCL are presented in Figure 4. In all 15 tumour samples hybridized with the appropriate probe sets, segregated signals (patterns C–H) were detected in 28–80% (mean 55.7%, standard deviation 15.3%) of the counted nuclei. The highest percentages (58–80%, mean 69.2%, standard deviation 8.9%) were found in five BL samples hybridized with the *MYC* breakpoint probe set, while in FL and MCL the segregation was found in 34–67% (mean 48.0%, standard deviation 14.6%) and 28–60% (mean 50.0%, standard deviation 13.5%) of the nuclei, respectively. For all probe sets, the counts were significantly higher than in control tissues ($p < 0.001$).The percentage of nuclei with the most stringent pattern indicative of a breakpoint, namely two colocalized and two segregated signals (Figure 4, BCL1, *BCL2*, and *MYC*, pattern C), varied from 13% to 49% (mean 28.0%, standard deviation 9.0%) in the 15 tumour samples. It was significantly higher than the threshold level (1.2%) observed in the negative controls ($p < 0.001$). Using the third algorithm, in which only the type of signal is evaluated (percentage of single and double, colocalized signals), without taking into account the distribution of signals in the cells), 40–70% (mean 53.9%, standard deviation 8.3%) single signals were detected in the tumour samples, as opposed to the threshold of 10.1% in the control samples ($p < 0.001$).

## Discussion

We describe the successful application of interphase FISH detection of three common chromosomal translocations in routine paraffin tissue sections. Small locus-specific probes were used, two of three probe sets already having been extensively tested by conventional interphase FISH analysis on fresh nuclei.

Currently, various FISH methods are used for the detection of chromosomal translocations in Interphase nuclei obtained from cell suspensions, imprints, or frozen tissue. Some studies have reported the successful application of this technique either on nuclei isolated from paraffin tissue blocks or on tissue sections of paraffin-embedded samples [8–14,29,30]. The major advantages of FISH on paraffin tissue sections are that the cells carrying the translocation can be analysed in a histological context and that less patients' material, including already available routine sections sent from elsewhere, can be used. There is a little control on the presence and number of tumour nuclei in the specimen during the procedure of isolating nuclei from tissue blocks, while paraffin tissue sections allow more supervised analysis. However, to the best of our knowledge, very few groups have been able to apply this technique in a routine diagnostic setting. The relatively low rate of success [8,9] may be related to a combination of factors including the small size of the targets, their degradation and/or masking during the fixation and further processing of the sample, and the use of relatively insensitive detection systems and fluorescence microscope equipment. Alternatively, large insert clones such as multi-yeast artificial chromosome (YAC) constructs (with a total length of hundreds of kb's) have been applied as locus-specific probes [13,14]. Another option is the implementation of probes against repetitive DNA targets, as they result in strong and easily detectable signals. Indeed, probes for

repetitive δ-satellite peri-centromeric DNA targets have been used both in combination with and without a locus-specific probe to detect chromosomal breakpoints in paraffin-embedded tissue [11,12]. Both the use of the large multi-YAC probes and the use of peri-centromeric probes have important disadvantages. Apart from the labour to deplete the YACs from repetitive DNA sequences to obtain relatively clean hybridization signals, the segregation and colocalization of such distant probes might be relatively fortuitous in interphase nuclei and give rise to ambiguous interpretation. Furthermore, neither of these approaches allows exact localization of the breakpoint, which is increasingly important since some genomic areas contain a high density of genes that might all be involved in a translocation. In contrast, the currently selected locus-specific probes allow detailed mapping of the breakpoints, as well as detection of small interstitial insertions and deletions (data not shown).

Individual optimization of the protease digestion time and/or enzyme concentration is often necessary to obtain relatively good results in paraffin tissue sections [11,29]. This is a time-consuming procedure and needs relatively large amounts of patient material. In contrast, in almost all samples tested so far, the currently used simple DNA demasking protocol resulted in reproducible signals, irrespective of the probe combinations or tissue samples. Some difficulties were encountered for Burkitt's lymphoma samples received from Africa, which might be due to excessively long fixation times or the use of other fixatives (data not shown). As the technique does not require special skills, consumables or equipment, and so far has been successfully applied to all three breakpoint regions on a consecutive series of more than 150 lymphoma and myeloma samples (Haralambieva *et al.*, in preparation), we believe that it can also be successfully applied to any other breakpoint.

Theoretically, the most informative approach to evaluating the interphase FISH results is to count all nuclei with all different patterns of signals. However, this is a very complex and time-consuming approach, since there are at least eight different patterns (Figure 3) to be scored and even more in the event of there being additional numerical chromosomal aberrations. Another disadvantage is that, in seeking to recognize all patterns in single nuclei, the observer tends to score at places where the tissue is less dense, such as at the edges of tissue sections. A second approach is to evaluate only the most stringent pattern (Figure 3, pattern C) and to omit the nuclei with an incomplete set of signals. This results in a very low cut-off level (1.2%) but the procedure remains time consuming and difficult, driving the investigator to a certain bias. Additionally, possible aneusomy must be considered. The third approach is to count the type of signals (colocalized against single) without assessing the nuclei. This is the most easy, non-selective approach, as it can be applied on the whole tissue section, even in areas with nuclear overlap, and since it is not influenced by aneusomy or other factors. In addition, the size of the tumour cells may also affect the performance of the test on tissue section; larger size obviously will result in more nuclei with an incomplete set of signals, because of the cutting artefacts. However, a high level of single split signals should promptly indicate the presence of translocation breakpoint. Single signals may also be caused by structural abnormalities different from translocation breakpoints, such as deletions in the target region). Obviously, these single signals must be discerned from single background signals, which indicates that the level of background signals must be assessed before counting.

In conclusion, we have shown that the detection of chromosomal breakpoints on paraffin tissue sections by interphase FISH can be reliably performed in a diagnostic setting. We did not, however, investigate samples with focal involvement of tumour cells, for instance trephine biopsies with foci of MCL or FL, nor did we test the sensitivity of our assays in samples involved by minimal residual disease.

***Acknowledgements***

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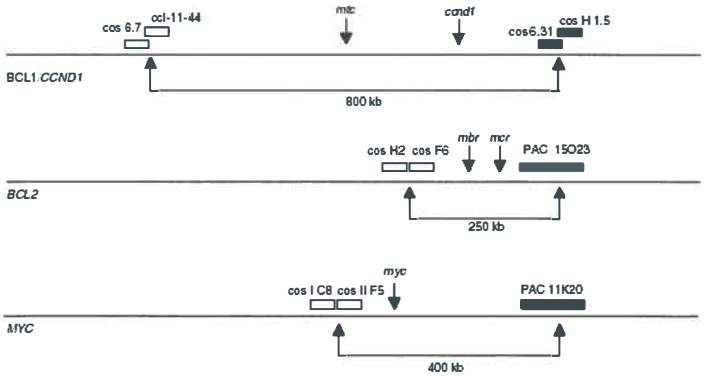


**Figure 1.** Schematic representation of the FISH probe sets for detection of the BCL1/*CCND1*, *BCL2* and *MYC* breakpoints. The probe sets were designed to flank the possible breakpoint regions as closely as possible, and their approximate positions are indicated on the map. They consist of differently labelled, in green (open bars) and red (dark bars), pooled cosmids and PACs.

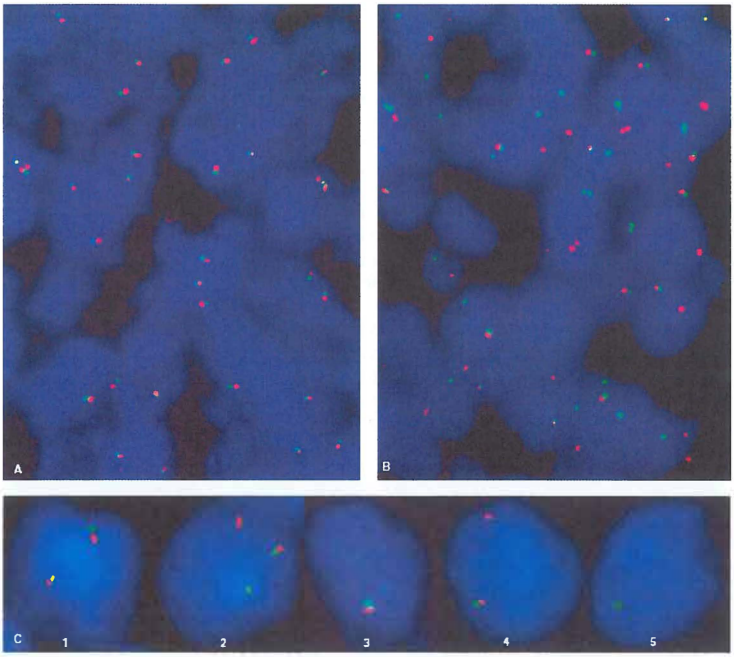


**Figure 2.** (A and B) Comparison of the distribution of FISH signals in paraffin sections of a negative control tissue sample and Burkitt's lymphoma, respectively, using alternatively labelled (red and green) *MYC* flanking probes in a FISH segregation assay. Only colocalized green and red signals are identified in the negative control samples (A), representing the intact *MYC* loci. In contrast, a *MYC* translocation breakpoint in a Burkitt's lymphoma is easily recognized by the presence of scattered single red and green signals (B). (C) Composite picture of cell nuclei, displaying different patterns of hybridization signals. 1: nucleus with 2 intact *MYC* loci, representing a reactive cell in the lymphoma sample; 2: nucleus with mono-allelic breakpoint, recognized by segregated red and green signals and a normal set of colocalized signals; 3–5: examples of the cutting artefacts resulting in nuclei with other signal patterns

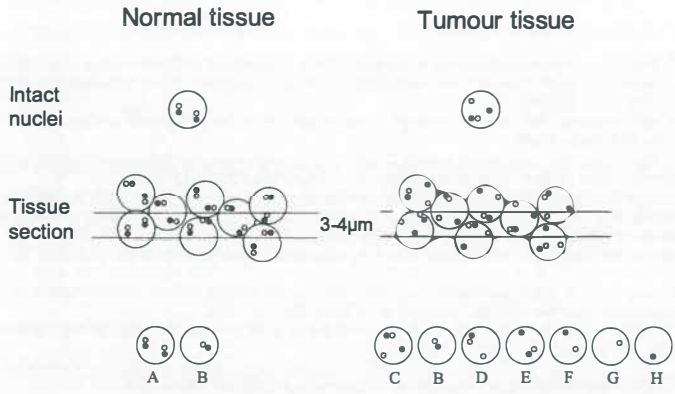


**Figure 3.** Schematic presentation of nuclei with different patterns of hybridization signals in tissue sections. Due to cutting artefacts, there are at least six more hybridization signal patterns to be evaluated in tissue sections than in intact interphase nuclei. (A–H) Different signal patterns in control tissue and in tumour samples with a mono-allelic chromosomal break. In tumour samples, pattern A represents the reactive cell compartment. Of note, more options must be considered in the event of numerical chromosomal aberrations

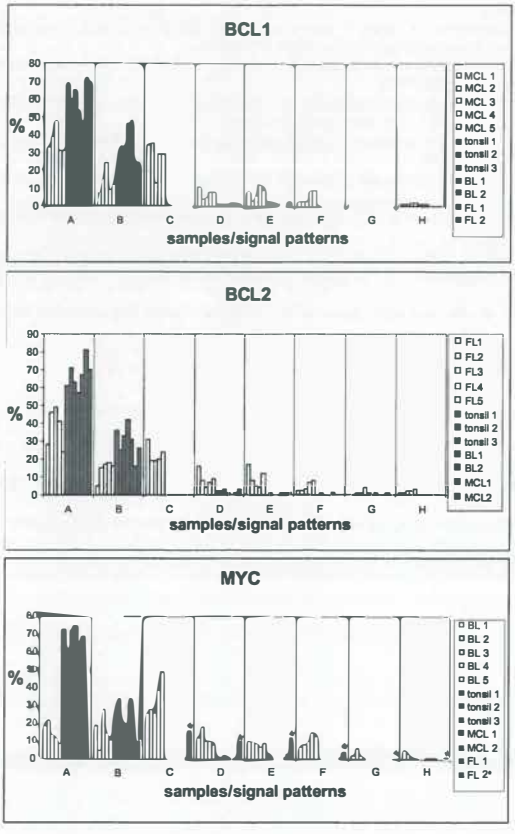


**Figure 4.** Scoring results in tumour samples and negative controls when hybridized with BCL1, *BCL2*, and *MYC* probe sets, as indicated in the boxes. (A–H) Different patterns as explained in Figure 3. The open and black bars represent the percentage of nuclei with a certain signal pattern in the tumour samples and negative controls, respectively. MCL: mantle cell lymphoma; FL: follicular lymphoma; BL: Burkitt's lymphoma. *A case of follicular lymphoma with both a *BCL2* and a *MYC* breakpoint

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## Chapter 4

# Interphase Fluorescence In Situ Hybridization for Detection of 8q24/MYC Breakpoints on Routine Histologic Sections: Validation in Burkitt Lymphomas from Three Geographic Regions

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A chromosomal translocation involving the *MYC* gene is characteristic of Burkitt lymphoma (BL) and represents a molecular disease marker with diagnostic and clinical implications. The detection of *MYC* breakpoints is hampered by technical problems, including the distribution of the breakpoints over a very large genomic region of approximately 1,000 kb. In this article, we report on the testing and validation of a segregation fluorescence in situ hybridization (FISH) assay for *MYC* breakpoints on a large series of BLs. A contig of overlapping genomic clones was generated, and two probe sets flanking the *MYC* gene were selected. Both probe sets were tested in an interphase FISH segregation assay on 8 B-cell lymphoma cell lines and 32 lymphoma samples with proved 8q24/*MYC* abnormalities and validated in 47 BLs from The Netherlands, Brazil, and Uganda. *MYC* translocation breakpoints were identified in 98% of the tumors of the test series and in 89% of the cases of the validation series. In 89% of all positive samples, the breakpoints were located between 190 kb 5' and 50 kb 3' of *MYC*. Nine cases had more distant breakpoints, and in one patient an insertion of *MYC* into the IGH region was detected. In two of the three BLs lacking CD10 expression, no breakpoint could be detected, suggesting that CD10 is a discriminative marker of BL. We did not find consistent differences between BL and atypical BL in incidence of an *MYC* breakpoint.

*Genes Chromosomes Cancer. 2004;40:10-18*

## Introduction

The eponym Burkitt lymphoma (BL) recognizes the unique contribution of Dennis Burkitt in describing the clinical features of this tumor found in children in equatorial Africa, delineating its geographical distribution, and pioneering its treatment (Burkitt, 1958). Currently, BL is believed to occur in three clinical settings: an endemic form (eBL), found in equatorial Africa and New Guinea; a sporadic (sBL), or "non-African," form, arising in nonendemic areas and a human immunodeficiency virus –associated form (HIV-BL; Wright, 1997; Diebold et al., 2001). Recognition and prompt diagnosis of BL are of clinical importance because it is a highly aggressive tumor that responds best to specific chemotherapeutic regimens that are different from those used for other aggressive B-cell lymphomas such as diffuse large B-cell lymphoma (DLBCL; Magrath et al., 1996; Harris et al., 1999; Mead et al., 2002). However, especially in (adult) patients outside Africa, the diagnosis of BL is not highly reproducible, even among experienced hematopathologists (Non-Hodgkin's Lymphoma Classification Project, 1997; Magrath et al., 2000). This is caused by the continuous histologic spectrum on which BL and DLBCL lie and by the partially overlapping histogenesis of both tumors. Ideally, the diagnosis of BL is based on clinical, morphologic, immunophenotypic, and cytogenetic criteria (Harris et al., 1994, 1999; Diebold et al., 2001). Indeed, the recent World Health Organization (WHO) classification recommended the presence of the t(8;14) or its variants t(2;8) and t(8;22) or of a *MYC* rearrangement as prerequisites for the diagnosis of BL and the morphologic variant, atypical Burkitt lymphoma (aBL; Diebold et al., 2001).

The translocations involving 8q24/*MYC*, which are a cytogenetic hallmark of BL (Zech et al., 1976; Dalla-Favera et al., 1982; Traub et al., 1982), result in deregulation of the *MYC* gene by juxtaposition to immunoglobulin regulatory sequences and loss or disruption of its 5' regulatory sequences. In about 85% of cases of BL, the translocation partner of *MYC* is the immunoglobulin heavy-chain (IGH) locus on chromosome 14, whereas, in the remaining 15%, the κ and λ light-chain loci on chromosomes 2 and 22, respectively, are involved. Furthermore, the position of the breakpoints in both the 8q24/*MYC* and the IGH loci varies with the geographic origin of the tumor (Pelicci et al., 1986; Shiramizu et al., 1990, 1991). In most non-African BLs, *MYC* breakpoints are within intron I or the region immediately 5' of *MYC*, whereas in non-African cases, they are dispersed over a large genomic area 5' of *MYC* (Joos et al., 1992a, b). Variant translocations usually involve the 3' region of *MYC* and also are dispersed over a large genomic area of approximately 300 kb (Henglein et al., 1989; Zeidler et al., 1994; Cario et al., 2000). Burkitt lymphomas in South American patients harbor a mixed pattern of chromosomal breakpoints (Gutierrez et al., 1992).

Although cytogenetic analysis certainly has the advantage of detecting not only t(8;14) and its variants but any other chromosomal aberration as well, this method is feasible only in a limited number of cases because it requires fresh tissue samples. Because of the variability of the breakpoints, polymerase chain reaction (PCR) is difficult to apply for regular diagnostic purposes. Long-distance PCR assays can detect t(8;14) in sporadic BL (Akasaka et al., 1996; Basso et al., 1999), but because of the position of the breakpoints, not in African BL.

Fluorescence in situ hybridization (FISH) is a preferable technique for detection of chromosomal breakpoints because this method allows the detection of widely dispersed breakpoints. Additionally, it can be applied on routinely processed tissue sections of a formalin-fixed, paraffin-embedded specimen and therefore allows studying large series of unselected cases (Haralambieva et al., 2002).

Several groups reported on the detection of *MYC* translocations in BL using FISH (Ried et al., 1992; Veronese et al., 1995; Davison et al., 1998; Rack et al., 1998; Siebert et al., 1998). However, the method has been tested on a limited number of cell lines and/or primary BL samples, and its application in the regular diagnosis of BL remains uncommon.

Here we report on a FISH segregation assay for detection of *MYC* translocation breakpoints in a genomic region from 900 kb 5' to 450 kb 3' of the gene. Two probe sets were selected on the basis of the available literature on *MYC* breakpoints (Pelicci et al., 1986; Henglein et al., 1989; Shiramizu et al., 1991; Gutierrez et al., 1992; Joos et al., 1992a, b; Zeidler et al., 1994; Cario et al., 2000) and designed for a FISH segregation assay. Both probe sets were tested on 8 B-cell lymphoma–derived cell lines and 32 lymphoma samples with cytogenetically or molecularly proved 8q24/*MYC* abnormalities and were validated on routinely processed tissue sections of 47 BL/aBLs collected from The Netherlands, Brazil, and Uganda, being representative of sporadic, South American, and equatorial African Burkitt lymphomas, respectively.

## Materials and Methods

***Test Series: cell lines and B NHL patients with an 8q24/MYC abnormality:*** Seven B-cell lymphoma–derived cell lines with a previously cytogenetically identified t(8;14) and the Namalwa cell line with a 8q24/*MYC* breakpoint that was detected by pulsed-field gel electrophoresis (Joos et al 1992b) were included. Daudi, Raji, Namalwa, Jijoye, and BL65 were derived from African BL patients, Ramos and BL41 were established from non-African BL patients, and Napi originated from a transformed follicular lymphoma (FL). All cell lines were grown in RPMI-1640 medium supplemented with 10% calf serum (In Vitrogen Life Technologies, Paisley, UK). BL65 was provided by Dr. G. M. Lenoir (IARC, Lyon, France). Ramos and Jijoye

were purchased from the American Type Culture Collection (Manassas, VA) and BL41, Daudi, and Raji from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). Napi was established at the Department of Hematology, Leiden University Medical Center (LUMC), Leiden, The Netherlands.

Routinely processed paraffin-embedded biopsy samples from 32 lymphoma patients were selected on the basis of previously cytogenetically proved 8q24 abnormalities and retrieved from the files of the Department of Pathology, LUMC, Leiden; the Department of Pathology & Laboratory Medicine, University Hospital Groningen (AZG); and the Department of Pathology, University Medical Center (AMC), Amsterdam, The Netherlands. The samples were classified according to the WHO classification scheme (Diebold et al., 2001) as BL ($N$ = 14), aBL ($N$ = 6), DLBCL ($N$ = 8), FL ($N$ = 3), or NHL not otherwise specified ($N$ = 1). One aBL and one DLBCL presented in HIV-positive patients. All cases had a (8;14)(q24;q32) except for 2 cases with a variant t(8;22)(q24;q11), one with t(2;8)(p12;q24), one with del(8)(q24), and one with add(8)(q24).

**Validation Series: Burkitt and atypical Burkitt Lymphomas:** Routinely processed, paraffin-embedded biopsy samples from 47 BLs and aBLs were retrieved from the files of the Departments of Pathology of the LUMC, AZG, and AMC, as well as from the Dutch Childhood Oncology Group, The Hague, The Netherlands; the Department of Pathology, Mekerere University, Kampala, Uganda; and the Division of Anatomic Pathology, Hospital Nossa Senhora das Gracas, Curitiba, Brazil. All samples were reviewed and classified according to the recent WHO classification (E.H., S.R., and P.K.). Only lymphoma samples with a high proliferative rate (Ki67 labeling index > 95%) were studied. Samples with centroblastic and/or immunoblastic morphology were classified as DLBCL and subsequently excluded. Four patients (N11, N32, N36, N38; Table 1) were HIV-positive.

**Fluorescence *in situ* hybridization:** To obtain appropriate FISH probes for the detection of breakpoints in the *MYC* region, we constructed a physical map by generating a contig of partially overlapping genomic clones in and around the *MYC* gene, using DNA fiber FISH (Fig. 1).

Clone pHM-1 (3129) represents a 10-kb *Eco*RI/*Eco*RI fragment in pBR322 and contains the whole *MYC* gene (kindly provided by Dr. I. Laird, Leiden University Medical Center, Leiden, The Netherlands). The P1 clones ICRF7000G569 (8034) and ICRF7000G2350 (8035), which were reported to cover and flank the *MYC* gene (Feo et al., 1994), were purchased from the Reference Library Database (Berlin, Germany). Cosmid clone K880 (4540; Mautner et al., 1995) covers the *MYC* gene and its 3' region; it was provided by Dr. J. Mautner, Institute of Clinical Molecular Biology and Tumor Genetics, Munich, Germany. PAC103G5 (4745), PAC117K22 (8071), and PAC124B14 (8072) were obtained from the RPCI-1 Human PAC Library of the Roswell Park Cancer Institute (available at the Leiden Genome Technology Center, Leiden, The Netherlands). PAC103G5 (4745) was identified by screening with the p8q290 probe from the *MYC*/BVR1 region, and both PAC117K22 (8071) and PAC124B14 (8072) with p8q373/550 probes from the *MYC*/PVT1 region (Henglein et al., 1989); probes were provided by Dr. M. Lipp, Ludwig-Maximilians-University, Munich, Germany. The cosmid clones cos-B11-I (4663), C11-I (4665), A5-II (4673), G11-I (4666), B10-III (4674), A7-I (4662), A12-II (4669), C10-II (4676), G12-I (4667), D12-II (4670), A1-I (4661), F9-II (4672), C8-I (4664), F5-II (4671), A7-II (4668), and C4-III (4675) were obtained by subcloning of partially digested DNA from YAC clone yP72 (8045), provided by Dr. C. Croce (Jefferson Cancer Institute, Philadelphia, PA; Veronese et al., 1995) in sCOGH6. The cosmid clones cos-H4.1, cos-P380J9, and cos-MYC72 (Siebert et al., 1998) were kindly provided by Dr. S. Joos (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The BAC clones RP11-414A9 and RP11-351C8 were selected according to their location in the minimal tiling path 5' of *MYC* by use of MapViewer at www.ncbi.nih.gov (build 33) and purchased from BACPAC, Children's Hospital Oakland (Oakland, CA).

DNA fiber FISH was used to map all genomic clones relative to pHM-1 (3129), K880 (4540), ICRF7000G569 (8034), and ICRF7000G2350 (8035). By comparing them with the already-established size markers for the 11q13 region (clones cos 6.22, cos 3.62, and cos 3.91, with a total length of 113.4 kb; Janssen et al., 2000) ascertained in cohybridization experiments, we determined the length of the combined signals of clones ICRF7000G2350 (8035), pHM-1 (3129), and PAC103G5 (4745) as 198.6 kb. These 3 clones were subsequently used as internal size markers for assessment of the length of the entire region.

Two probes for the IGH locus were selected for the *MYC*/IGH colocalization FISH assay. Cosmid cos Ig6 covers the constant region of the immunoglobulin heavy-chain locus (Vaandrager et al., 1998) and was provided by Dr. T. Rabbits (MRC Center, Cambridge, UK). PAC27M16 (5093), located immediately 3' of the immunoglobulin heavy-chain locus (manuscript in preparation), was provided by Dr. D. Cox (Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada).

To achieve easily interpretable results on tissue sections, a two-color FISH segregation assay was designed, using breakpoint flanking probes that were labeled differently (Fig. 1) by standard nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (Roche, Basel, Switzerland). Hybridization on DNA fibers, on interphase nuclei from frozen samples, and on routine paraffin tissue sections was performed as described previously (Vaandrager et al., 1996, 2000; Haralambieva et al., 2002). To obtain sufficient hybridization efficiency on some of the paraffin-embedded samples from Uganda, additional digestion by increasing the pepsin concentration (up to 5.0%) and incubation time (up to 1hr) was necessary.

Three hyperplastic tonsils, 2 mantle cell lymphomas, and 2 FLs without a *MYC* breakpoint were used as negative controls. The cut off level for every probe set was determined for the percentage of cell nuclei with at least a single segregated signal (Haralambieva et al., 2002, method 1) in these 7 negative controls and inferred from the mean + 3 standard deviations. For this purpose, 200 nuclei were counted. Samples with a segregated signal level above the cut off level were considered positive for a breakpoint.

**EBER *in situ* hybridization:** In situ hybridization was performed for detection of EBER 1-2 mRNAs using a fluorescein-conjugated EBV (EBER) peptide nucleic acid probe (Dako, Glostrup, Denmark) on paraffin tissue sections. Alkaline phosphatase– conjugated anti–FITC sheep IgG Fab fragments (Roche) were used in a second step. The visualization was performed with 5-bromo-4-chloro-3-indolyl-phosphatase, 4-nitroblue tetrazolium (Roche, Mannheim, Germany), and MgCl2.

**Immunohistochemistry:** Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue samples. Briefly, 4-µm sections were heat-retrieved for antigen in a buffer solution of 50 mM Tris and 2 mM EDTA, pH 9, for 30 min in a microwave oven. The following markers were analyzed: CD 20 (clone L26, dilution 1:100; Dako), Ki-67 (clone MIB-1, dilution 1:100; Dako), CD10 (clone 56C6, dilution 1:20; Novocastra, Newcastle upon Tyne, UK), BCL2 (clone 124, dilution 1:25; Dako), and BCL6 (PG-B6p, dilution 1:20; Dako). Immunostaining was performed in a Nexes automated immunostaining machine (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions.

For BCL6, an additional amplification step was incorporated into the standard immunostaining protocol. A tumor was considered positive for expression of specific antigen if more than 10% of the tumor cells showed appropriate immune reactivity.

A percentage of the Ki-67-positive cells was determined as the actual percentage of tumor cells, thus excluding reactive macrophages and small lymphocytes.

## Results

**Selection of probes for detection of breakpoints in *MYC* region by interphase FISH:** All clones were mapped relative to each other by FISH on DNA fiber preparations of reactive tonsils. The total area covered by the contig was estimated to be more than 1.3 Mb, ranging from 900 kb 5' to 450 kb 3' of *MYC* (Fig. 1A; see Materials and Methods section).

For detection of the breakpoints in the immediate 5' region (intron I of *MYC)* and in the 3' region, we selected two pooled cosmids, [C8-I & F5-II] and PAC 1117K22 (Fig. 1A). According to published data, this probe set should detect most breakpoints of non-African BL and BL with a variant translocation. To detect the breakpoints in the far 5' region as reported in African BL, we selected an additional probe set (cosmids [B11-I & C11-I] and PAC 103G5; Fig. 1A). All breakpoints within a region from 190 kb 5' to 50 kb 3' of *MYC* should be detected by both probe sets (Fig. 1A). In the 7 negative control tissues, segregated signals were detected in 1%–5% (mean 4.2%; *SD* 1.5) and 1%–7% (mean 3.3%; *SD* 1.9) of nuclei when hybridized with the 3' and 5' probe sets, respectively. The cut off level for both probe sets was set at 9.0%.

**Using two selected probe sets in a FISH segregation assay to detect *MYC* breakpoints in a series with proved 8q24/*MYC* abnormalities:** *MYC* breakpoints were identified in 39 of 40 (98%) samples with a cytogenetically or molecularly proved 8q24/*MYC* breakpoint. In 36 of 39 (92%) of the positive samples, including all BL cell lines, the breakpoint was identified by segregation of both probe sets; therefore, the breakpoints were in a region that went from 190 kb 5' to 50 kb 3' of *MYC*. Three cases [one with a t(8;14) and two with a t(8;22)] had a breakpoint more than 50 kb 3' of *MYC*. In case 26, a DLBCL with a cytogenetically proved t(8;14), no breakpoint was detected with either FISH probe set. Because the breakpoint might have been outside the range of the probe sets, we used an additional probe, BAC RP11-351C8, which maps 900 kb 5' of *MYC*, in combination with the pooled cosmids [B11-I & C11-I]. Again, no breakpoint was detected. In addition, two probes for *IGH* at 14q32 (cosIg6 and PAC27M16) did not colocalize with any of the *MYC* probes.

**Detection of *MYC* breakpoints by segregation FISH in randomly selected BLs:** We validated both probe sets on 47 BLs: 15 BLs from Uganda, 10 BLs from Brazil, and 22 BLs from The Netherlands. No cytogenetic data were available for these patients. A *MYC* breakpoint was identified in 41 of the 47 BLs, which represented 86% of the Dutch cases, 70% of the BLs from Brazil, and 100% of the BLs from Uganda (Table 1, Fig. 1B). In 35 of the 41 breakpoint-positive cases (85%), the translocation breakpoints were in a genomic region that ranged from 190 kb 5' to 50 kb 3' of *MYC*. Three far-3' and 3 far-5' breakpoints were detected in 1 case from The Netherlands, 2 cases from Brazil, and 3 cases from Uganda.

In 6 cases, no breakpoint was detected either with both FISH segregation assays or with the above-mentioned additional far-5' probe, BAC RP11-351C8 (Table 1). Unfortunately, case 16 could not be studied further because of lack of available tissue. In the 5 remaining cases, we used an additional probe, ICRF7000G569, which covers the *MYC* gene, in combination with the *MYC* region flanking probes. In 1 of

the 5 cases - a BL from The Netherlands (case 14, Table 1) - we observed colocalization of the *MYC* flanking probes (PAC117K22 and the pooled [C8-I & F5-II]), but 3 signals for probe ICRF7000G569. As assessed in independent FISH experiments, one of these 3 signals did not colocalize with either 117K22 or [C8-I & F5-II]. This indicates that a part of the region, including the *MYC* gene itself, was excised from its original location at 8q24. An additional FISH assay using probe ICRF7000G569 and two IgH probes (cosIg6 and PAC27M16) showed colocalization of this third ICRF7000G569 signal with both IGH signals, indicating that *MYC* was inserted in the IGH region at 14q32.

**Presence of *MYC* breakpoints in the context of histopathologic, epidemiologic, and clinical data:** The results of immunostaining, EBER in situ hybridization, FISH analysis of *MYC* breakpoints, and the patients' data are summarized in Table 1.

The patients were between 3 and 52 years old, with a median age of 8 years. Most BLs (71%) presented at extranodal sites, especially the ileocecum/abdomen. In 4 patients from Uganda, the tumors were in the head and neck region. Ten cases were classified as aBL. As compared to the findings in classical BL, aBL did not show any significant difference in clinical presentation, immunophenotype, EBV association, or *MYC* breakpoints (Table 1). All BLs were BCL6-positive, 93% stained for CD10, and 98% were BCL2-negative. EBER in situ hybridization for EBV was positive in 52% of all tumors: 38% of the Dutch, 93% of the Ugandian, and 50% of the Brazilian cases. We did not find any association of the incidence and location of *MYC* breakpoints with age (child vs. adult), EBV status, and site of presentation (extranodal vs. nodal). However, 2 of 3 CD10-negative BLs, both from Brazil, did not harbor any *MYC* breakpoint. All 5 BL cases without a detectable *MYC* breakpoint represented children: 2 from The Netherlands and 3 from Brazil. Only 2 of these patients had an extranodal tumor, and none had an ileocecal tumor. The single EBV-positive BL without an *MYC* breakpoint was from an HIV-positive patient from Brazil (Table 1, case 32). As indicated above, 2 of the BLs without a detectable *MYC* breakpoint showed an aberrant immunophenotype with absence of CD10 expression.

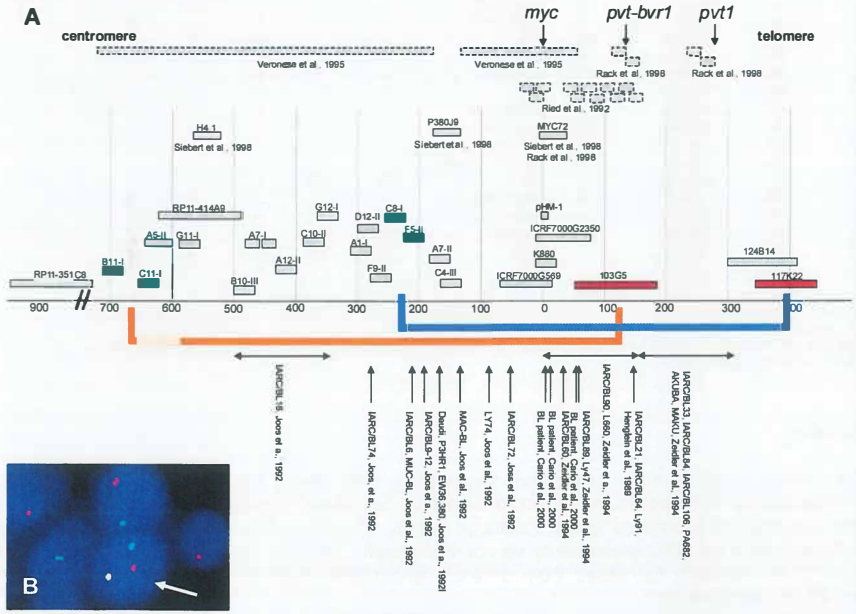


**Figure 1. A**: Schematic presentation of the *MYC* gene region. The arrows indicate the position of *MYC*, *PVT1* (plasmocytoma variant translocation 1), *BVR1* (Burkitt variants' rearranging region 1), and the approximate positions of most of the reported breakpoints (which we located relatively far from the *MYC* gene). All genomic clones (bars) were mapped by DNA fiber FISH relative to pHM-1, K880, ICRF7000G569, and ICRF7000G2350, which all cover the *MYC* gene. Subsequently, two probe sets were selected for interphase FISH segregation assay: (1) cosmids C8-I and F5-II (pooled) and PAC 1117K22, also indicated by a blue connection line; and (2) cosmids B11-I and C11-I (pooled) and PAC 103G5, indicated by an orange connection line. The green and red bars represent the probes labeled with digoxigenin and biotin, respectively. In the upper part of the figure, probes previously used in interphase FISH are indicated (Ried et al., 1992; Veronese at al., 1995; Rack et al., 1998; Siebert et al., 1998). The bars with an interrupted outline represent the probes not mapped by us by fiber FISH but located according to the reported data. The multi-YAC constructs (Davison et al., 1998, Zymed) and LSI *MYC* dual-color break-apart rearrangement probes (Vysis) are not shown in this figure because they map to a very large genomic region on both sides of *MYC* (Zymed) or have been incompletely mapped accurately (Vysis). **B**: Example of FISH signal patterns in paraffin tissue section of a Burkitt lymphoma, using the B11-I and C11-I probe set and 103G5, as indicated in A. The translocation breakpoint is easily recognized by the presence of single red and green signals. The arrow indicates a cell with a set of colocalizing signals (yellow spot), representing the normal allele, and one single red and one green signal, indicating the presence of a monoallelic break. Note that because of the sectioning artifacts, many cells have incomplete sets of FISH signals.

TABLE 1. FISH for 8q24/*MYC* Breakpoints in Randomly Selected BLs/aBLs

| Number | Diagnosis | Source | Age | Site | CD10 | Bcl2 | EBV | *MYC* 3′ | *MYC* 5′ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | BL | Netherlands | 13 | omentum | + | − | − | + | + |
| 2 | BL | Netherlands | 9 | mesentery | + | − | + | + | + |
| 3 | BL | Netherlands | 13 | abdomen | NA | − | − | + | + |
| 4 | BL | Netherlands | 5 | abdomen | + | − | − | + | + |
| 5 | aBL | Netherlands | 10 | ileocecum | + | − | − | + | + |
| 6 | BL | Netherlands | 13 | ileum | + | − | − | + | + |
| 7 | BL | Netherlands | 7 | ileocecum | + | − | − | + | + |
| 8 | BL | Netherlands | 6 | prostate | + | − | − | + | + |
| 9 | BL | Netherlands | 39 | lymph node | + | − | + | + | + |
| 10 | BL | Netherlands | 52 | lymph node | + | − | + | + | + |
| 11[a] | BL | Netherlands | 26 | lymph node | + | − | + | + | + |
| 12 | BL | Netherlands | 15 | ileocecum | NA | NA | NA | + | + |
| 13 | BL | Netherlands | NA | NA | NA | NA | NA | + | + |
| 14 | BL | Netherlands | 12 | abdomen | + | − | + | −ins | −ins |
| 15 | BL | Netherlands | 8 | ileocecum | + | − | − | + | + |
| 16 | BL | Netherlands | 6 | lymph node | + | − | − | − | − |
| 17 | BL | Netherlands | 8 | ileocecum | − | − | NA | + | + |
| 18 | aBL | Netherlands | 9 | oropharynx | + | − | NA | − | − |
| 19 | aBL | Netherlands | 9 | ileocecum | + | − | NA | + | − |
| 20 | BL | Netherlands | 7 | abdomen | + | + | + | + | + |
| 21 | aBL | Netherlands | 7 | tonsil | + | − | NA | + | + |
| 22 | BL | Netherlands | 5 | tonsil | + | − | − | + | + |
| 23 | aBL | Brazil | 4 | periorbital | − | − | − | − | − |
| 24 | BL | Brazil | 5 | abdomen | + | − | + | + | + |
| 25 | BL | Brazil | 21 | abdomen | + | − | − | + | + |
| 26 | aBL | Brazil | 3 | retroperitoneum | + | − | + | + | + |
| 27 | BL | Brazil | 10 | lymph node | − | − | − | − | − |
| 28 | aBL | Brazil | 4 | abdomen | + | − | + | + | + |
| 29 | aBL | Brazil | 3 | lymph node | + | − | − | + | − |
| 30 | aBL | Brazil | 3 | retroperitoneum | + | − | + | − | + |
| 31 | BL | Brazil | 8 | lymph node | + | − | − | + | + |
| 32[a] | BL | Brazil | 5 | lymph node | + | − | + | − | − |
| 33 | BL | Uganda | NA | NA | + | − | NA | − | + |
| 34 | BL | Uganda | 13 | mandible | + | − | + | + | + |
| 35 | BL | Uganda | 9 | abdomen | + | − | + | + | + |
| 36[a] | BL | Uganda | 7 | NA | + | − | + | + | + |
| 37 | BL | Uganda | 22 | lymph node | + | − | + | + | + |
| 38[a] | BL | Uganda | 23 | lymph node | + | − | − | + | + |
| 39 | BL | Uganda | 7 | abdomen | + | − | + | + | + |
| 40 | BL | Uganda | 4 | mandible, maxilla | + | − | + | + | + |
| 41 | BL | Uganda | 5 | NA | NA | NA | + | + | + |
| 42 | aBL | Uganda | 35 | lymph node | + | − | + | + | + |
| 43 | BL | Uganda | 15 | lymph node | + | − | + | + | + |
| 44 | BL | Uganda | 20 | mandible | NA | NA | + | − | + |
| 45 | BL | Uganda | 3 | orbit | NA | NA | + | + | + |
| 46 | BL | Uganda | 6 | NA | + | − | + | + | − |
| 47 | BL | Uganda | 15 | abdomen | + | − | + | + | + |

BL—Burkitt lymphoma; aBL—morphologic variant atypical Burkitt lymphoma; [a]HIV-positive patients; NA—data are not available; ins—insertion of *MYC* into the *IgH* region.
The data on B-cell markers, BCL6 and Ki67 are not included in the table because all tumors had a B-cell phenotype, expressed BCL-6, and the MIB-1/Ki-67 labeling index was > 95%.

## Discussion

In the present study, a novel interphase segregation FISH assay for the detection of 8q24/*MYC* translocation breakpoints was applied, and it could detect an 8q24/*MYC* breakpoint in 98% of the cases in the test series and 89% of the cases in the validation series. Therefore, this assay provides a rapid and reliable tool for detection of *MYC* breakpoints on routine paraffin sections of BL samples. Moreover, the assay appears to be valuable for cases from different geographic regions known to harbor differently positioned 8q24/*MYC* breakpoints.

Various FISH strategies have been used to detect *MYC* translocation breakpoints. One approach is to use an IGH probe in combination with a single 8q24 probe that spans the breakpoint region. In a translocation, the 8q24 probe will split, and one signal will colocalize with the IGH probe (Veronese et al., 1995; Siebert et al., 1998). However, the size of the additional 8q24 signal depends on the position of the breakpoint; in some instances, it can be too small to be visualized. In addition, cutting artifacts and the overlap of nuclei in tissue sections generate both loss of signals and fortuitous overlap, resulting in patterns that are difficult to recognize and interpret. We used a more appropriate assay, a simple segregation assay in which differently colored breakpoint-flanking signals segregate upon chromosomal breakage.

According to the limited data available from DNA pulsed-field and FISH mapping studies (Joos et al., 1992a, b), most t(8;14) breakpoints in African BLs are spread over a region of 55–340 kb 5'of *MYC*. Breakpoints 3'of *MYC* involved in the variant translocations have been mapped from 16 to 250 kb 3' of *MYC* within the *BVR1* and *PVT1* regions (Henglein et al., 1989; Cario et al., 2000).

However, previously reported FISH assays were designed to detect breakpoints in specific 5' or 3' regions, not to detect all possible breakpoints (see Fig. 1; Ried et al., 1992; Veronese et al., 1995; Rack et al., 1998; Siebert et al., 1998). Two FISH assays for the detection of *MYC* breakpoints are commercially available through Zymed (Zymed Laboratories, South San Francisco, CA) and Vysis (Vysis, Inc., Downers Grove, IL). The Zymed assay consists of six yeast artificial chromosome (YAC) constructs flanking the *MYC* region that have been depleted of repetitive DNA sequences by subtraction technology (Davison et al., 1998; Haralambieva et al., 2003). The three centromeric and three telomeric YACs cover a region of approximately 1.5 and 4.5 Mb, respectively, and leave a gap of approximately 1 Mb around *MYC*. Because of this design, breakpoints can only be detected but not mapped by this assay. In the assay from Vysis, the 3' probe starts approximately 1 Mb 3' of *MYC* and covers a region of 400 kb. However, the exact location of the 5' probe is not known, and according to the manufacturer, it starts not farther than 140 kb 5' of *MYC*. Consequently, the assay may miss breakpoints within the far-5'area (cases 30, 33, and 44, as well as additional cases of non-African BL with such far-5' breakpoints; see Table 1; Haralambieva et al., in preparation). Furthermore, in other BLs (e.g., the Daudi cell line), the break might be within the region covered by the probe itself and therefore would give rise to a complex signal pattern. In contrast to both commercially available probe sets, our small locus-specific probes (cosmids and PACs) are relatively simple to handle and allow a relatively exact mapping of the breakpoints, as well as detection of small interstitial insertions and deletions.

Thus far, all of the methods of detecting *MYC* breakpoints, including the commercially available assay from Vysis, have been validated in relatively few BL cell lines and primary BL specimens (Ried et al., 1992; Veronese et al., 1995; Davison et al., 1998; Rack et al., 1998; Siebert et al., 1998; Basso et al., 1999). According to our results in the current test and validation series, the majority (89%) of all 8q24/*MYC* breakpoints are within a region from 190 kb 5' to 50 kb 3' of *MYC*, whereas 11% of the breakpoints are far-5' or -3' breakpoints. In keeping with previous reports (Henglein et al., 1989; Joos et al., 1992a,b; Cario et al., 2000), the latter may be relatively more common in African BL patients and BL with a variant breakpoint (Table 1). Of interest is the position of the breakpoint in one Dutch BL with a cytogenetically proved t(8;14) that was located between 50 and 350 kb 3' of *MYC*. This configuration indicates that not only the variant translocations but also t(8;14) breakpoints can be localized far-3' of *MYC*.

Six cases (in the test and validation series) did not show a breakpoint using our FISH assay. Recently, Fabris et al. (2003) reported on 8q24/*MYC* breakpoints in multiple myeloma cell lines and primary myeloma samples. Interestingly, 5 of the 11 cell lines had a breakpoint more than 2.7 Mb from *MYC*; however, it is not clear whether *MYC* or other genes were affected in these cases. In addition to the possibility of such distant breakpoints, *MYC* can also be deregulated by other mechanisms, such as mutations, small insertions of Ig regulatory elements into the *MYC* locus, and insertion of *MYC* into the Ig loci. Indeed, we encountered one BL with an insertion of *MYC* into the IgH region, which, to our knowledge, has not been described previously. Of note, such small insertions will remain undetected by conventional cytogenetics and most FISH assays and can only be detected by specifically designed FISH assays. Although 8q24/*MYC* translocation breakpoints are considered a cytogenetic hallmark of BL, some early cytogenetic studies (Zech et al., 1976; Komblau et al., 1991) included a considerable number of BLs negative for t(8;14) or its variants. Further analysis of such *MYC* breakpoint–negative cases is necessary, especially to investigate whether they are true BLs or belong to a "gray zone" between BL and diffuse large B-cell lymphoma. Our data suggest that the absence of CD10 expression, a bona fide marker of germinal-center B cells, is associated with the absence of an 8q24/*MYC* breakpoint, indicating that these CD10-negative tumors do not belong to the category of BL. Atypical BLs did not differ from classical BL in any aspect. Thus, in keeping with the current WHO classification (Diebold et al., 2001), these aBLs probably belong to the entity of true BL.

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# Chapter 5

## Clinical, Immunophenotypic, and Genetic Analysis of Adult Lymphomas with Morphologic Features of Burkitt Lymphoma

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A prompt distinction of Burkitt lymphoma (BL) versus diffuse large B cell lymphomas (DLBCL) has important clinical implications; however, this distinction can be difficult. We analyzed 74 adult gray zone and 10 reference pediatric BL using immunohistochemistry (Ki-67, CD10, bcl2, bcl6) and fluorescence in situ hybridization (FISH) for *MYC*, *BCL2*, and *BCL6* breakpoints. Two algorithms for classification were followed: algorithm A used a two-step review by four hematopathologists and algorithm B a set of only biologic markers (Ki-67 ≥ 90%, CD10+, bcl6+, bcl22, *MYC* breakpoint+, *BCL2* and *BCL6* breakpoint -). Both algorithms categorized all reference cases as BL. In the adult group, algorithm A resulted in 21adult BL and 52 DLBCL and algorithm B in 23 BL and 51 "non-Burkitt" lymphomas (nBL); 9 cases (12%) contained two different translocations and were categorized as nBL in algorithm B. Fifteen cases (20%) fulfilled the BL criteria of both algorithms. Although not considered as BL according to both algorithms, many other lymphomas showed nonetheless a phenotypic and/or genetic shift to BL. BL according to algorithm B was more homogeneous with respect to clinical presentation (gender and localization) than BL defined by algorithm A. Our data suggest that only a few cases of these gray zone lymphomas represent true de novo BL. Immunohistochemistry for Ki-67, CD10, and bcl2 with analysis of *MYC* and preferably also *BCL2* and *BCL6* may be advised as a marker panel for this diagnostic dilemma.

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## Introduction

The REAL and WHO classifications introduced the concept of clinicopathologic entities in which histology, immunophenotype, genetic data, as well as clinical features are integrated.[16,19] This led to a major increase in interobserver and intraobserver agreement, as well as in a worldwide high level of acceptance by hematologists and oncologists.[17] However, a few problems remained, one being the differential diagnosis of Burkitt (BL), atypical BL (aBL), Burkitt-like lymphoma (REAL classification), and a subset of diffuse large B-cell lymphomas (DLBCL) with features of BL. Indeed, this is a long-standing problem, already starting with the introduction of the term "undifferentiated non-Burkitt's lymphoma" in the Rappaport classification and "small non-cleaved non-Burkitt's lymphoma" in the Lukes' classification.[25,33] In a key manuscript on the reproducibility and clinical impact of the REAL classification, an unacceptable low interobserver and intraobserver agreement existed with respect to the histologic distinction between Burkitt-like lymphoma and DLBCL, which did not improve by integration of immunohistology or clinical data.[42]

From a biologic point of view, BL seems to be a relatively homogeneous disorder characterized by the expansion of early germinal center blasts in the so-called dark zone of normal germinal centers.[29] In parallel to their normal counterparts, BL cells almost invariably express CD10 and bcl6 but lack bcl2.[11] Genetically, BLs carry a reciprocal translocation t(8;14)(q24;q32) or one of its variants resulting in deregulation of *MYC*. Three subtypes of BL have been described: endemic (equatorial Africa and parts of New Guinea), sporadic, and HIV-associated BL, which differ in their clinical presentation and association with Epstein-Barr virus (EBV) infection.[6,18,27,38] These subtypes, although uniformly characterized by *MYC* translocations, occasionally show subtle to marked cytomorphologic variation (eg, plasmacytoid differentiation in some HIV-associated BLs).[19] In addition, although cytogenetically indistinguishable, both the 8q24 and 14q32 chromosomal breakpoints differ at the molecular level in endemic and sporadic BL patients.[27,31,32,34] This suggests that different mechanisms are involved in the generation of chromosomal breakpoints (eg, IGH hypermutation and IGH class switch recombination, respectively) and therefore that endemic and sporadic subtypes probably originate from different stages of B-cell development.

DLBCL is much more heterogeneous and can be molecularly divided in at least two subtypes: lymphomas with a germinal center B cell-like profile of gene expression (GCB profile) and lymphomas that express genes more related to activated B cells (ABC profile).[2,10,12] Also from a cytomorphologic point of view, DLBCL is highly heterogeneous with a range from Hodgkin-like features in T-cell-rich B-cell lymphoma to cases composed of relatively small- to medium-sized cells that mimic BL.[16,19] At the cytogenetic and molecular genetic level, a large number of chromosomal translocations can be found, including *BCL6* breakpoints in approximately 20% to 35%, *BCL2* breakpoints in 15% to 20%, and *MYC* breakpoints in 5% to 10% of the tumors. Furthermore, many tumors have complex karyotypes with multiple structural abnormalities.[9,20,22]

The presence of a diagnostic overlap between BL and DLBCL resulted in the introduction of poorly defined borderline categories such as undifferentiated non-BL, small noncleaved non-BL, Burkitt-like lymphoma (BLL), and aBL.[3,16,19,39] These terms generated considerable confusion and were differently used by various hematopathologists. However, a prompt diagnosis and distinction of BL versus DLBCL bear important clinical implications since treatment of BL often differs from treatment of DLBCL patients.[28] The present WHO classification recommends the presence of 8q24 breakpoints and/or *MYC* rearrangement as a prerequisite to the diagnosis of BL and its morphologic variants. Unfortunately, genetic aberrations involving *MYC* are not strictly disease specific and, to the best of our knowledge, this WHO recommendation has not been validated in a large lymphoma series.

Here, we describe the clinical, phenotypic, and molecular features of 74 lymphomas obtained from adult patients with either a typical BL or a gray-zone BL/DLBCL as well as 10 pediatric reference BL cases. All cases were analyzed for cytomorphology, phenotype, and translocation breakpoints within the 8q24/*MYC*, 18q21/*BCL2*, and 3q27/*BCL6* breakpoint regions (further called *MYC*, *BCL2*, and *BCL6*). Two different algorithms for classification were followed, one conventional algorithm accomplished through a two-step review procedure by four hematopathologists and the other by means of application of exclusively a combination of immunophenotypic and genetic markers.

## Material and methods

***Selection of cases:*** Seventy-four cases collected between 1990 and 2003 (Table 1) were retrieved from the archives of the Departments of Pathology of the University Medical Center Groningen (N = 23) and the Department of Pathology of the University of Würzburg (N = 33) as well as from a review of two clinical trials on aggressive NHL in The Netherlands (HOVON 27 and HOVON 40; N = 18). All cases were lymphomas from adult patients (age>16 years); all tumors included as Burkitt lymphoma were non-endemic cases; AIDS-related lymphomas were excluded. The following criteria were used to include cases in the present study:

- Cases diagnosed as BL with a typical growth pattern and a high proliferation rate as established by staining for Ki-67/MIB-1 (in general, >90% positive nuclei), however, occasionally with a relatively high degree of nuclear polymorphism (BL and aBL in the WHO classification)

- Cases submitted as DLBCL with features of BL, in particular a composition of relatively small- to medium-sized blasts, a high degree of monomorphism, a cohesive growth pattern, a high proliferation rate, and/or a starry-sky appearance. Classic DLBCL were not included in the study.
- Cases for which the hematopathologist could not decide upon the differential diagnosis of BL and DLBCL.

Ten cases of unambiguous pediatric BL (Table 1, cases A–J) were included as a reference group.

***Immunohistochemistry:*** Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections, after 30 minutes antigen heat-retrieval in 50 mM Tris/2 mM EDTA buffer solution, pH 9.0 in a microwave oven. The following markers were analyzed: Ki-67 (clone MIB-1, dilution 1:100; DAKO, Glostrup, Denmark), CD10 (clone 56C6, dilution 1:20, Novocastra, Newcastle upon Tyne, United Kingdom), bcl2 (clone 124, dilution 1:25, DAKO), and bcl6 (PG-B6p, dilution 1:20, DAKO). Immunostaining was performed in the Nexes automated immunostaining machine (Ventana Medical systems, Tucson, AZ) according to the manufacturer's instructions. For Bcl6 an additional amplification step was incorporated into the standard immunostaining protocol. A tumor was considered to be positive for expression of a specific antigen if more than 10% of the tumor cells showed appropriate immune reactivity. A percentage of the Ki-67-positive cells was determined as an actual percentage of the tumor cells, thus excluding reactive background cells.

***Fluorescence in situ hybridization:*** All cases were studied by segregation FISH on paraffin tissue sections for the presence of translocation breakpoints within three breakpoint regions, *MYC*, *BCL2*, and *BCL6*.

For detection of *MYC* translocation breakpoints, three probe sets were used15 as follows: all cases were tested with the probes flanking the region from 190 kbs 5'to 450 kbs 3' of *MYC*. Samples that did not show any breakpoint were studied with an additional probe set covering the far (700 kbs) area upstream of *MYC*. The remaining negative cases were tested with a probe combination designed to detect a possible excision of *MYC* from 8q24 and subsequent insertion elsewhere in the genome.[15]

*BCL2* breakpoints were studied using two previously reported probe sets, for detection of mbr/mcr and 5'/FVT1 breakpoints, as well as excision of *BCL2*.[37]

The FISH segregation assay for detection of 3q27/*BCL6* region breakpoints will be described in detail elsewhere. Briefly, one probe set flanks the MBR in the 5' noncoding region of *BCL6* (PAC 133M19 and PAC 165I21, kindly provided by Dr. R. Dalla Favera, Columbia University, NY). The second probe set consists of PAC 133M19 and BAC RP11-137K3 and detects the ABR breakpoint region located between 245 and 285 kbs 5' of *BCL6*.[8] BAC RP11-137K3 was selected on basis of its location in the minimal tiling path 5' of *BCL6* (MapViewer at www.ncbi.nih.gov; build 33) and obtained from BACPAC Resources, Oakland, CA).

The probes were labeled using Kreatech's ULS nonenzymatic labeling method (Kreatech Diagnostics, Amsterdam, The Netherlands, *BCL2* and *MYC* probes) or by standard nick-translation (*BCL6* probes) with biotin-16-dUTP or digoxygenin-11-dUTP (Roche, Basel, Switzerland). The conditions for the hybridization and the evaluation of FISH results on routine tissue sections have been described recently.[13]

***Histopathologic classification:*** To reach a diagnosis according algorithm A, all samples, including the 10 reference BL cases, were reviewed by four hematopathologists (H.K.M.-H., G.O., P.M.K., and E.H.) in a two-step procedure (Tables 1 and 2). First, each pathologist evaluated the samples independently, based on the histology, immunohistochemistry, age, and site of the involvement, but without knowledge of molecular data, the original diagnosis, and/or the opinion of other reviewers. All cases were categorized according to the WHO classification scheme; however, the interpretation of the weight of the individual morphologic and immunophenotypic features was left to the judgment of the individual reviewer. aBL referred to the cases with atypical cytomorphologic features, mostly a considerable variation in nuclear and nucleolar size, irregularities of the nuclear contour, or plasmacytoid differentiation, nevertheless considered to belong to the BL entity. As a second step, all four reviewers discussed the discordantly classified cases using a multiheaded microscope, and this resulted in a final diagnosis further called "algorithm A diagnosis." After this review, individual cases were discussed with knowledge of the molecular data, but the algorithm A diagnosis was not altered.

The second approach (algorithm B) included direct application of a combination of markers, recommended by the WHO classification for the diagnosis of BL. Since the primary selection of the cases was based on morphology (see selection of cases) and, therefore, all cases were considered to represent gray zone lymphomas, any additional histopathology review was omitted. According to this algorithm, BL referred only to these cases that had a Ki-67 labeling rate of>90%, that were bcl6+, CD10+, and bcl2-, and carried a *MYC* breakpoint. All cases that did not fulfill these criteria, but also the cases that harbored additional *BCL2* and/or *BCL6* breakpoints and therefore might represent transformed lymphomas, were excluded from the category of BL and denoted as non-Burkitt lymphoma (nBL).

***Statistical analysis:*** For comparison of the various parameters the Fisher exact test was applied.

**Results**

The classification of all the samples together with the patients' data, immunophenotype, and the molecular data (presence of breakpoints in *MYC*, *BCL2*, and *BCL6*) are listed in Table 1 and summarized in Table 3.

**TABLE 1. DATA OF ALL PATIENTS**

| case | Sex | age | site | Algorithm A | Algorithm B | Ki67 | CD10 | bcl2 | bcl6 | FMYC | FBCL2 | FBCL6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | m | 10 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| B | m | 9 | parapharynx | BL | BL | 99% | + | - | + | + | - | - |
| C | m | 4 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| D | m | 5 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| E | m | 6 | prostate | BL | BL | 99% | + | - | + | + | - | - |
| F | m | 7 | ileocecum | aBL | BL | 99% | + | - | + | + | - | - |
| G | f | 9 | retrop., mediastinum | BL | BL | 99% | + | - | + | + | - | - |
| H | m | 13 | ileocecum, mesentery | BL | BL | 99% | + | - | + | + | - | - |
| I | m | 13 | intestine, retrop., pleural c. | BL | BL | 97% | NE | - | + | + | - | - |
| J | m | 13 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| 1 | f | 39 | nasal cavity | BL | BL | 99% | + | - | + | + | - | - |
| 2 | m | 71 | liver, lymph node | aBL | BL | 95% | + | - | + | + | - | - |
| 3 | f | 20 | lymph node | BL | BL | 99% | + | - | + | + | - | - |
| 4 | m | 18 | stomach, lymph node | aBL | BL | 99% | + | - | + | + | - | - |
| 5 | f | 71 | thyroid | aBL | BL | 95% | + | - | + | + | - | - |
| 6* | f | 33 | ovary | aBL | BL | 97% | + | - | + | + | - | - |
| 7* | m | 34 | mediastinum | BL | BL | 99% | + | - | + | + | - | - |
| 8 | m | 39 | lymph node | BL | BL | 95% | + | - | + | + | - | - |
| 9 | m | 52 | lymph node | aBL | BL | 99% | + | - | + | + | - | - |
| 10 | m | 68 | lymph node | BL | BL | 95% | + | - | + | + | - | - |
| 11 | m | 68 | mesenteric lymh node | aBL | BL | 99% | + | - | + | + | - | - |
| 12 | m | 62 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| 13 | m | 22 | lymph node, waldeyer | aBL | BL | 95% | + | - | + | + | - | - |
| 14* | m | 40 | ileum | BL | BL | 99% | + | - | + | + | - | - |
| 15 | m | 34 | ileocecum | BL | BL | 99% | + | - | + | + | - | - |
| 16 | m | 52 | oral cavity | DLBCL | BL | 99% | + | - | + | + | - | - |
| 17 | m | 24 | soft tissues, BM | DLBCL | BL | 95% | + | - | + | + | - | - |
| 18 | m | 32 | ileocecum | DLBCL | BL | 99% | + | - | + | + | - | - |
| 19* | m | 43 | stomach | DLBCL | BL | 99% | + | - | + | + | - | - |
| 20 | f | 46 | breast, BM | DLBCL | BL | 99% | + | - | + | + | - | - |
| 21 | f | 67 | stomach | DLBCL | BL | 90% | + | - | + | + | - | - |
| 22 | m | 74 | lymph node | DLBCL | BL | 99% | + | - | + | + | - | - |
| 23* | m | 42 | stomach | DLBCL | BL | 99% | + | - | + | + | - | - |
| 24 | f | 65 | lymph node | BL | nBL | 90% | + | + | - | + | - | - |
| 25 | m | 63 | omentum majus | BL | nBL | 99% | + | + | + | + | - | - |
| 26* | m | 65 | omentum | aBL | nBL | 95% | + | - | + | - | - | - |
| 27 | m | 33 | lymph node | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 28 | f | 61 | stomach | DLBCL | nBL | 80% | + | + | - | - | - | - |
| 29 | m | 79 | lymph node | DLBCL | nBL | 95% | + | - | + | - | - | - |
| 30 | m | 38 | stomach | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 31 | m | 76 | colon | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 32 | m | 78 | lymph node | DLBCL | nBL | 99% | + | + | + | - | - | - |
| 33 | f | 71 | soft tissues | DLBCL | nBL | 99% | - | + | | - | - | - |
| 34 | f | 48 | lymph node | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 35 | m | 29 | lymph node | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 36* | m | 36 | ileum | DLBCL | nBL | 50-99% | + | - | + | - | - | - |
| 37 | f | 65 | pancreas, peritoneum, ln | DLBCL | nBL | 50-99% | + | + | NE | + | - | - |
| 38* | f | 65 | spleen | DLBCL | nBL | 80% | - | + | NE | - | - | + |
| 39 | f | 90 | lymph node | DLBCL | nBL | 90% | - | + | + | - | - | - |
| 40* | f | 58 | lymph node | DLBCL | nBL | 97% | - | + | + | - | - | +ABR |
| 41* | f | 60 | ovary | DLBCL | nBL | 90% | + | + | + | - | - | - |
| 42* | m | 60 | skin | DLBCL | nBL | 99% | - | + | + | - | - | - |
| 43* | m | 44 | lymph node | DLBCL | nBL | 95% | + | + | NE | - | - | - |
| 44 | m | 52 | lymph node | DLBCL | nBL | 95% | - | + | + | - | + | - |
| 45 | f | 63 | breast | DLBCL | nBL | 95% | - | + | + | - | - | + |
| 46 | f | 65 | lymph node | DLBCL | nBL | 99% | + | + | + | - | - | - |

| case | Sex | age | site | Algorithm A | Algorithm B | Ki67 | CD10 | bcl2 | bcl6 | FMYC | FBCL2 | FBCL6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 47 | m | 66 | lymph node | DLBCL | nBL | 70% | - | + | + | - | - | + |
| 48 | m | 67 | small bowel | DLBCL | nBL | 95% | - | + | + | - | - | - |
| 49 | m | 69 | nasopharynx | DLBCL | nBL | 90% | - | + | NE | - | - | - |
| 50 | f | 69 | cecum | DLBCL | nBL | 90% | + | + | + | - | + | - |
| 51 | f | 72 | lymph node | DLBCL | nBL | 90% | - | + | + | - | - | - |
| 52 | f | 80 | lymph node | DLBCL | nBL | 90% | + | + | NE | - | + | - |
| 53 | m | 84 | lymph node | DLBCL | nBL | 99% | - | + | + | - | - | - |
| 54 | m | 89 | skin | DLBCL | nBL | 95% | -CD5+ | + | - | + | - | - |
| 55 | m | 62 | lymph node | DLBCL | nBL | 95% | - | + | + | - | - | - |
| 56 | m | ? | bone | DLBCL | nBL | 80% | - | + | + | - | - amp | + |
| 57 | f | 52 | lymph node | DLBCL | nBL | 90% | - | + | + | - | - | + |
| 58 | f | 53 | lymph node | DLBCL | nBL | 95% | - | + | + | + | - | - |
| 59 | ? | 41 | stomach | DLBCL | nBL | 90% | + | + | - | - | - | NE |
| 60 | m | 47 | ileum | DLBCL | nBL | 90% | + | - | + | - | - | + |
| 61* | f | 19 | ovary | DLBCL | nBL | 98% | + | - | + | - | - | - |
| 62 | m | 48 | tonsil | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 63* | m | 64 | lymph node | DLBCL | nBL | 99% | + | - | + | - | - | - |
| 64 | m | 13 | lymph node | DLBCL | nBL | 90% | + | + | - | + | - | - |
| 65 | f | 63 | abdominal | DLBCL | nBL | 95% | + | + | + | + | - | - |
| 66* | f | 58 | lymph node | aBL | nBL | 99% | + | + | + | + | - | +ABR |
| 67* | m | 36 | abdomen | aBL | nBL | 95% | + | + | + | + | + | - |
| 68 | f | 43 | ovaries, small bowel, CNS | aBL | nBL | 99% | - | + | + | + | - | + |
| 69 | f | 56 | mesentery | DLBCL | nBL | 90% | + | - | + | + | + | - |
| 70 | f | 50 | lymph node | DLBCL | nBL | 90% | + | + | + | + | + | - |
| 71* | m | 44 | omentum | DLBCL | nBL | 50-90% | + | + | + | + | + | - |
| 72 | f | 52 | ovary | DLBCL | nBL | 85% | + | + | + | + | + | - |
| 73* | m | 64 | abdomen | DLBCL | nBL | 97% | + | + | + | + | - | + |
| 74 | f | 33 | lymph node | DLBCL&foll | nBL | 99% | + | - | + | - | + | +ABR |

*: cases included in HOVON clinical trials. NE: not evaluable; Case 56 represented an adult patient (>16 yr) without known date of birth; ABR: breakpoint in the ABR of *BCL6*. [8]cases 36, 37 and 71 showed heterogeneous Ki 67 labeling pattern ranging from 50 to 99% in different part of the sample

***Two diagnostic algorithms to diagnose Burkitt lymphoma***

Algorithm A diagnosis: as a result of the conventional histopathology review procedure that included the multihead review session, all 10 reference pediatric BL and 21 adult cases (28%) were diagnosed as BL (typical BL, n = 10; aBL, n = 11); 52 adult cases were classified as DLBCL (70%). One DLBCL harbored foci of follicular lymphoma grade 3B and was classified as "other" (Tables 1 and 3). In 58% of the cases, a consensus diagnosis was reached independently by all four pathologists and in 80% by three of four pathologists. As shown in Table 2, the individual pathologists differed in their preference to use or avoid the diagnosis BL: reviewer 2 diagnosed BL in 81% of the cases finally diagnosed as such but showed a concomitant trend to overdiagnose it in the DLBCL cases (7 of 52 cases; 11%, data not shown), whereas reviewer 4 was relatively reluctant to diagnose BL at all. A consensus diagnosis by all four pathologists was reached independently in only 24% (5 of 21) of the adult BL and in 69% (36 of 52) of the DLBCL. As expected, a complete consensus was much more easily to achieve in the pediatric BL (80%).

Classification according to the algorithm B resulted in 23 BL and 51 nBL. Applying the algorithm A diagnosis on these 23 BL cases, 8 of had been classified as BL, 7 as aBL, and 8 as DLBCL. Therefore, 71% of these exclusively phenotypically and genetically defined BL presented with an atypical morphology, and even 35% would have been placed in a different lymphoma category using conventional microscopy. In reverse, 6 cases (29%) that had been classified as BL or aBL according to algorithm A did not fulfill the BL criteria of algorithm B. In 2 cases, this was caused by a weak bcl2 expression of the tumor cells, in 1 case by the lack of a detectable 8q24/*MYC* breakpoint and in 3 cases (50%) by the presence of a second translocation (see below).

**Table 2. Consensus in histopathological diagnosis (algorithm A)**

| consensus diagnosis | N | Reviewer 1 (%) | Reviewer 2 (%) | Reviewer 3 (%) | Reviewer 4 (%) | Consensus by 3/4 pathologists (%) | Consensus by 4/4 pathologists (%) |
|---|---|---|---|---|---|---|---|
| (a)BL pediatric | 10 | 100 | 100 | 90 | 90 | 90 | 80 |
| (a)BL adult | 21 | 62 | 81 | 52 | 48 | 48 | 24 |
| DLBCL | 52 | 88 | 84 | 88 | 98 | 94 | 69 |
| Other | 1 | 100 | 100 | 0 | 0 | 0 | 0 |
| Total adult | 74 | 81 | 84 | 77 | 82 | 79 | 55 |

***Clinical and immunohistologic data:***

All data available on the patients and their tumors, categorized according to the two algorithms, are listed in Table 1and summarized in Table 3. Both algorithms showed a male predominance, which tended to be more pronounced in the BL group according to algorithm B than any other category (difference between both algorithms, not significant). BL patients were significantly younger than DLBCL and nBL patients, and especially according to algorithm B, BL patients more often presented with extranodal disease ($P < 0.05$). All BLs but also the majority of the DLBCLs and nBLs were CD10 and bcl6 positive, indicating the limited value of these markers in this gray zone area. Most of all BLs showed a strong uniform bcl6 expression pattern resembling that of a normal germinal center, while nBLs/DLBCLs tended to label more heterogeneously, with variation of the intensity and percentage of positive tumor cells (data not shown). In contrast to the BLs in which bcl2 protein expression was infrequent (24% according to algorithm A and by definition absent according to algorithm B), 62% of the DLBCLs and 73% of the nBLs expressed bcl2 protein at an extent of 10% or more tumor cells (Table 3).

***Molecular data***

A *MYC* breakpoint was detected in a high proportion (57%) of the tumors, ie, by definition of algorithm B in all BL, but also in 29% of the nBL (difference significant, $P < 0.001$; Tables 1 and 3). Using algorithm A, differences in translocation frequencies were less explicit but still significantly different (Table 3). Most *MYC* breakpoints were detected with the probe set covering breakpoints in a region of 190 kb 5' to 450 kb 3'of MYC, and only three additional breakpoints were exclusively detected with the second probe set for breakpoints more 5' of MYC (data not shown). In 2 cases, additional FISH studies with a third probe set supported the presence of an insertion of MYC instead of a translocation (data not shown).

*BCL2* breakpoints were detected in 9 samples: 3 nBLs (algorithm B) carried this breakpoint as a single genetic abnormality, whereas in 5 other nBLs it was combined with a *MYC* breakpoint (see below). In one nBL with foci of follicular lymphoma grade 3B, it was combined with a *BCL6* breakpoint (Table 1).

*BCL6* breakpoints were identified in 10 tumors: in 6 nBLs it was the only genetic lesion, in 3 nBLs it was combined with a *MYC* breakpoint, and in the above mentioned "composite" nBL it was combined with a *BCL2* breakpoint.

Further analysis showed a significant positive association between CD10 expression and the presence of a *MYC* translocation breakpoint ($P < 0.001$), whereas *BCL6* breakpoint-positive samples tended to lack CD10 ($P = 0.05$) and to express bcl2 ($P < 0.05$). Bcl2 expression did not correlate with a *BCL2* breakpoint since only 7 of the 37 cases (19%) that expressed the protein contained a *BCL2* breakpoint. Neither was bcl2 expression a strong predictor for the absence of a *MYC* breakpoint: 14 of 37 cases (38%) with expression of bcl2 contained a *MYC* breakpoint. Since almost all cases in this series were bcl6 positive, no correlation of antigen expression with any genetic lesion could be recognized.

**Table 3. Immunophenotype, molecular genetic results and clinical data according to the different classification algorithms**

| | | *Algorithm A* | | *Algorithm B* | |
|---|---|---|---|---|---|
| | BL pediatric N=10 | (a)BL N=21 | DLBCL N=52 | (a)BL N=23 | nBL N=51 |
| age range (yrs)<br>median | 4-13<br>9 | 18-71<br>43 | 13-90<br>60 | 18-74<br>42 | 13-90<br>63 |
| m/f ratio | 9,0/1,0 | 2,0/1,0 | 1,3/1,0 | 2,8/1,0 | 1,1/1,0 |
| extranodal presentation (%) | 10 (100) | 9 (43) | 25 (48) | 15 (65) | 19 (37) |
| consensus pathologists (%) | 8 (80) | 5 (24) | 36 (69) | - | - |
| range Ki67 labeling (%) | 97-99 | 95-99 | 50-99 | 95-99 | 50-99 |
| CD10+ (%) | 10 (100) | 20 (95) | 35 (67) | 23 (100) | 33 (65) |
| bcl6+ (%) | 10 (100) | 20 (95) | 43/47 (91) | 23 (100) | 40/46 (89) |
| bcl2+ (%) | 0 (0) | 5 (24) | 32 (62) | 0 (0) | 37 (73) |
| CD10+,bcl6+,bcl2- (%) | 10 (100) | 15 (71) | 8 (15) | 23 (100) | 0 (0) |
| *any MYC* breakpoint (%) | 10 (100) | 20 (95) | 18 (35) | 23 (100) | 15 (29) |
| *MYC* breakpoint alone (%) | 10 (100) | 18 (86) | 13 (25) | 23 (100) | 7 (14) |
| *BCL2* breakpoint (%) | 0 (0) | 1 (5) *MYC* | 7 (13); 4 *MYC* | 0 (0) | 9 (18); 5 *MYC* |
| *BCL6* breakpoint (%) | 0 (0) | 2 (10); 2 *MYC* | 8 (16); 1 *MYC* | 0 (0) | 11/50 (22); 3 *MYC* |

One case of composite lymphoma (DLBCL and follicular lymphoma grade IIIB) was omitted from DLBC (algorithm A) but included in the nBL group (algorithm B)

***Samples with double translocation***

Nine samples carried a combination of translocation breakpoints in *MYC*, *BCL2*, and/or *BCL6* (Table 1). In keeping with the classification criteria of algorithm B, all cases were categorized as nBL; however, the group was heterogeneous according to algorithm A: one tumor with a *MYC* and *BCL2* breakpoint was classified as BL and 4 as DLBCL, 2 lymphomas with a combination of a *MYC* and *BCL6* breakpoint were diagnosed as BL and one as DLBCL (Fig. 1C), and finally one lymphoma with a *BCL2* and *BCL6* breakpoint was diagnosed as a composite DLBCL and follicular lymphoma, grade 3B. The majority (6 of 9) of these patients were female and 6 of 9 tumors coexpressed CD10, bcl2, and bcl6. Of interest, in 2 of 3 cases with a *MYC* and *BCL6* breakpoint, the *BCL6* breakpoint was located within the alternative breakpoint region (ABR) of *BCL6*, while in 5 of 6 DLBCLs with a single *BCL6* breakpoint, it was located in the major breakpoint region (MBR). Furthermore, all cases with this double breakpoint expressed bcl2 protein.

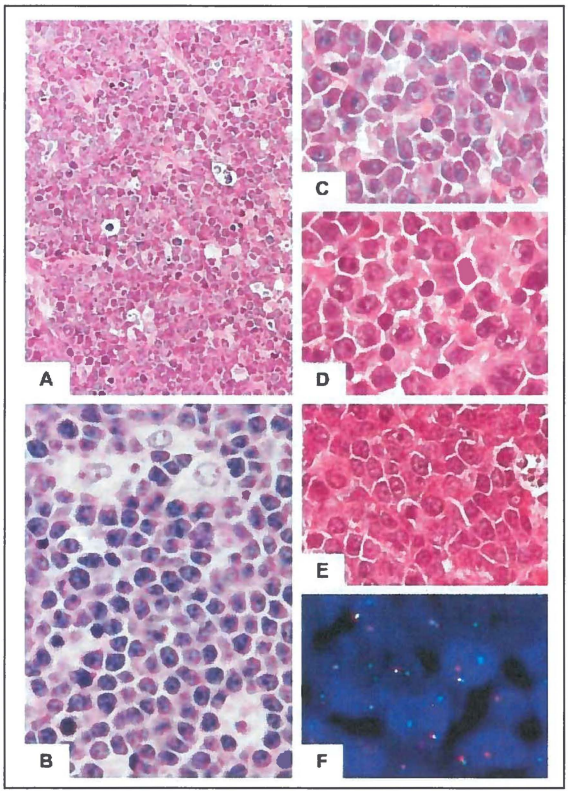


**FIGURE 1.** Morphology of Burkitt lymphomas and DLBCL. **A,** A low power overview at 40x magnification. **B–E**, The detailed morphologic spectrum of various cases at a magnification of 100X (oil immersion). Details on clinical presentation are presented in Table 1. **A**, Tumor no. 16 (51 years, oral cavity), algorithm A DLBCL, algorithm B BL, with a "starry sky" appearance, relatively small cell composition, and high degree of monomorphism, features shared by most of the cases included in this study. The tumor showed expression of CD10 and bcl6 without bcl2 and with exclusively a breakpoint in the *MYC* region. **B**, Tumor no. 4 (18 years, tumor stomach), algorithm A aBL, algorithm B BL, with a subtle degree of nuclear polymorphism, expression of CD10 and bcl6 without bcl2 and with exclusively a breakpoint in the *MYC* region. **C**, Case no. 68 (43 years, tumor of both ovaries) algorithm A aBL, algorithm B nBL, with an even higher degree of nuclear polymorphism, that co-expressed bcl6 and bcl2 protein but lacked CD10 and contained a double translocation in the *MYC* and *BCL6* regions. **D**, Case no. 18 (32 years, ileocecum), algorithm A DLBCL, algorithm B BL, containing tumor cells with abundant cytoplasm, and large nuclei with prominent eosinophilic nucleoli and an "open" chromatin pattern reminiscent of centroblasts and immunoblasts; this case showed the immunophenotype and genotype of BL: CD10 and bcl6 expression, with lack of bcl2 and a breakpoint in *MYC* with no other breakpoints. Note that this tumor presented as an ileocecal mass reminiscent of BL. **E**, Case no. 62 (48 years, tumor of the tonsil), algorithm A DLBCL, algorithm B nBL, with nuclei that are neither typical of BL nor DLBCL, that co-expressed CD10 and bcl6, without bcl2, but did not contain any detectable *MYC* or other breakpoint. **F**, One example of fluorescent in situ hybridization with *MYC* probes with colocalization of one set of probes and segregation of the other set of probes indicating a monoallelic *MYC*/8q24 breakpoint.

## Discussion

We report on the combined molecular and immunohistochemical analysis of lymphomas that represent a histologic gray zone area between classic BL and DLBCL. Since we could not relate our data to any established gold standard, two algorithms for classification of the samples were compared. One algorithm attempted to make the distinction between BL and DLBCL with conventional morphologic and immunohistochemical means, including a careful two-step pathology review procedure. The second algorithm aimed at separating BL from all other lymphomas (collectively labeled as nBL) and made use of purely immunophenotypic and genetic criteria. Since submission of the cases to the study was already based on a primary morphologic selection (see Materials and Methods), the second algorithm omitted any further pathology review.

In general, the great majority of DLBCL can be reliably distinguished from BL, and the diagnostic problem only represents relatively rare borderline cases composed of small to medium-sized tumor cells with a high proliferation rate, a "starry-sky" appearance, and a cohesive growth pattern.

Most likely due to a lack of firm histologic and phenotypic criteria and ubiquitously applicable molecular markers, the lack of a real gold standard as well as the continuously changing and confusing nomenclature, few informative publications are available on this issue.[1,3,7,14,16–18,28,30] Certain studies made an attempt to link the borderline categories of "Burkitt-like lymphoma" and "small non-cleaved, non-Burkitt's lymphoma" to DLBCL or BL and/or generated data on their biologic and clinical heterogeneity.[7,14,30] A recent report on 13 Burkitt-like lymphomas suggested that they represent a subcategory of classic BL and differ both biologically and clinically from DLBCL.[7] However, the DLBCL samples of this study were randomly chosen while our DLBCL specimens were selected on the basis of morphologic similarities with BL. In keeping with previous reports, our study confirms the lack of robust histologic criteria since all four hematopathologists independently reached the same final diagnosis in only 55% of all submitted cases (algorithm A).

Using algorithm B, 31% of the adult lymphomas were classified as BL. Interestingly, 8 of these 23 BL cases (35%) had been assigned DLBCL according to algorithm A. Their exclusion from BL was mainly based on the nuclear pleomorphism with the presence of centroblasts and/or immunoblasts among smaller more classic BL blasts. Vice versa, 6 of 21 (29%) lymphomas categorized as (a)BL by algorithm A should have been removed to the nBL category using algorithm B. This was due to the presence of a double (*MYC* and *BCL2* or *BCL6*) breakpoint (3 cases), lack of a detectable *MYC* breakpoint (1 case), and/or an immunophenotype thought to be incompatible with BL (5 cases). In particular, bcl2 expression (>10% of

tumor cells positive) was frequently seen in these cases (5 of 6 cases). The combined application of both algorithms on the total group of adult gray-zone lymphomas resulted in a group of only 15 cases (20%; case N 1–15, Table 1) that, similar to the 10 pediatric cases, fulfilled all morphologic, phenotypic, and genetic criteria of aBL. Apart from the more robustness of the criteria, also some clinical data suggest that the definition of BL according to algorithm B might be slightly superior to that of algorithm A.

In particular, more patients were male and presented with extranodal disease, both characteristics of pediatric and endemic BL as well. Since our study is retrospective in nature and obviously consisted of a mixture of patients who not only differed in treatment but also in clinical factors with well known independent prognostic impact on outcome in either BL and DLBCL, we could not use clinical outcome, eg, overall survival, to compare the different groups as defined by both algorithms. This was also impossible for the 18 patients who were retrieved from two clinical trials with high-dose, short duration chemotherapy. Such an analysis would have required a much larger group of patients.

A breakpoint within the *MYC* region was detected in all but one aBL as defined by algorithm A, indicating that the t(8;14) or variant translocation is associated with a certain morphology and phenotype. However, we frequently encountered this breakpoint in the remaining lymphomas as well (35% in our subset of DLBCLs according to algorithm A and 29% in the nBL group of algorithm B). Therefore, this marker should not be used alone but only in combination with other meaningful markers. Similarly, Au et al reported on a large spectrum of B-cell tumors carrying cytogenetically proven 8q24 aberrations, including BL, BLL, and (transformed) lymphomas such as follicular and mantle cell lymphomas as well.[3]

Apparently, even in the cases not diagnosed as BL by both algorithms, our primary selection of lymphoma cases had already resulted in a biased shift of phenotypic and genetic characteristics toward BL. For instance, nBL diagnosed according to algorithm B expressed CD10 in 65% of the cases instead of the expected 20% to 45% in regular DLBCL. Similarly, almost all cases expressed bcl6 protein. This shift was also evident by the relatively low frequency of *BCL6* breakpoints (22% instead of 30%–40% as expected on basis of literature data for regular DLBCL) and the high incidence of *MYC* breakpoints. The reported frequencies of *MYC* breakpoints in DLBCL vary between 4% and 15% depending on the methods used (cytogenetics, FISH, Southern blot, or long distance PCR).[1,5,9,19,22,24,26,36] The FISH probes we used flank a large genomic region as compared with Southern blot or long distance PCR; however, this cannot fully explain the current high frequency of MYC breakpoints in DLBCL or nBL. In a separate study on 62 International Prognostic Index (IPI) high risk but further unambiguous DLBCL in which we applied the same (FISH) methods, 18% had a *MYC* breakpoint, 18% a *BCL2* breakpoint, and 27% a *BCL6* breakpoint (Boerma EJ, personal communication).

Eight tumors in adult patients contained a combination of *MYC* and *BCL2* and/or *BCL6* breakpoints. The combination of *MYC* and *BCL2* breakpoints has been extensively described.[21,26,35,40] Lymphomas and leukemias carrying both breakpoints probably develop by a two-step transformation with first the generation of the t(14;18) in a precursor B-cell and thereafter a break at 8q24/*MYC* that mostly occurs at the mature B-cell stage. Although sometimes morphologically similar to aBL, these tumors should be distinguished from true BL with a sole break in *MYC* as they often represent a very aggressive disease with a poor clinical outcome. Tumors with a concomitant *MYC* and *BCL6* breakpoint have been reported occasionally; however, the clinical and biologic significance of these alterations has not been established.[1,26,36] In the present series, the 3 cases with a *MYC* and *BCL6* breakpoint shared morphologic and immunophenotypic similarities with lymphomas carrying double breakpoints in *MYC* and *BCL2*, including expression of bcl2 protein. Interestingly, in 2 of the 3 samples, the 3q27 breakpoint was located within the ABR of 3q27/*BCL6* that is predominantly involved in follicular lymphoma.[8] Whether such tumors represent transformed (follicular) lymphomas or are part of true BL remains to be established.

Partly based on the expression of CD10 and bcl6, Burkitt lymphoma is considered as a neoplastic proliferation of (early) germinal center B cells. Since many DLBCL and nBL of our study also expressed CD10 and bcl6, also these lymphomas might be supposed to be derived from germinal center B cells. However, the frequent expression of bcl2 protein in 62% of the DLBCL and 73% of the nBL cases without a concomitant underlying t(14;18) is not compatible with a germinal center B-cell phenotype. Our data indicate that immunohistochemistry of bcl2 protein expression may be a surrogate marker for *BCL2* and *BCL6* breakpoints. Based on this observation and the fact that bcl6 protein analysis has little additional value to CD10, we suggest that immunohistochemistry for Ki-67, CD10, and bcl2 in combination with FISH analysis for *MYC* breakpoints could be a minimum marker panel to be used for the diagnosis of BL in adult patients. Additional FISH analysis for *BCL2* and *BCL6* breakpoints might be advisable as well. Future, more extensive gene expression studies,[2,4,23] but also genome-wide genetic studies such as comparative genomic hybridization or array comparative genomic hybridization, in combination with well-controlled clinical trials are indispensable to define the exact entity of adult BL and to learn which adult patients might benefit from specific therapeutic regimens.

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## Chapter 6

# Florid Granulomatous Reaction in Epstein-Barr Virus-positive Nonendemic Burkitt Lymphomas
## *Report of Four Cases*

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Epithelioid cell granulomas have been reported in association with a wide range of neoplasms including malignant lymphomas. In lymphoma, this refers mainly to Hodgkin disease and T-cell derived non-Hodgkin lymphomas where a granulomatous reaction is probably evoked by aberrant cytokine production in the tumor cells or other cells composing the tumor background. Here we report on four cases of sporadic Burkitt lymphoma with unusual florid granulomatous reaction. In all samples, the tumor cells were admixed with numerous epithelioid cells that formed clusters and granulomatous lesions.

No microorganisms could be detected at the tissue level, and there were no clinical or laboratory indications of an underlying immunodeficiency. The lymphomas harbored a specific morphology and immunophenotype of Burkitt lymphoma, and the presence of translocation breakpoint in *MYC* gene was confirmed by Interphase fluorescence in situ hybridization. In all four patients, the lymphoma was associated with Epstein-Barr virus infection, detected by EBER in situ hybridization and the latency I phenotype as defined by lack of expression of LMP1. All four patients were treated with polychemotherapy, achieved a complete remission, and are alive without evidence of disease. We draw attention to this unusual phenomenon as it caused difficulties in interpretation and delayed diagnosis and hypothesize on the possible role of Epstein-Barr virus products.

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## Introduction

In rare tumors, including malignant lymphomas, epithelioid granulomas may be encountered.[4–8] The mechanism underlying this phenomenon remains poorly understood; however, some authors favor a view that it represents some form of local response to the tumor.[6]

Clusters of epithelioid cells are not uncommon in peripheral T-cell non-Hodgkin lymphomas [20–23] and may be related to an aberrant cytokine production. For some categories, such as the lymphoepithelioid cell lymphoma (Lennert's lymphoma), they represent a characteristic histologic feature of the tumor.[8,17] This phenomenon is relatively common in Hodgkin lymphoma, both in the primary tumor and at sites that are not involved by the tumor.[1,7,14,22] Several reports suggested that the granulomatous reaction represents a nonspecific host response to the tumor and therefore it might account for a more favorable clinical outcome of these patients.[22] In some instances, epithelioid granulomas may also be evoked by an associated infection (eg, mycobacteria but also other bacteria, viruses, fungi, or protozoa),[4,6] which is not uncommon in states of an underlying, tumor-associated, or therapy-related immunodeficiency. Of note, local irritants may evoke a similar tissue reaction and also sarcoidosis can coexist with the lymphoma.[4,5,7]

Occasionally a granulomatous reaction was noted in B cell lymphomas,[3,9,15] with two reports on Burkitt lymphoma (BL).[11,13] Here we report on four cases of sporadic BL associated with an extensive granulomatous reaction. All four cases contained Epstein-Barr virus (EBV), which suggest a pathogenic role of this virus in the formation of the granulomatous lesion.

## Material and Methods

All four samples were retrieved from the pathology files of the Department of Pathology and Laboratory Medicine, University Hospital Groningen, Department of Pathology, Isala Clinics, Zwolle and Department of Pathology, University Medical Center Amsterdam, Amsterdam, The Netherlands. All four cases have been collected during the histologic revision of the samples included in a separate study on BL (N = 67) from Europe, South America, and equatorial Africa (Haralambieva at al, in press).

All samples were fixed in 4% buffered formalin and processed for routine histopathologic examination. Ziehl-Neelsen, periodic acid-Schiff, and Grocott stainings were performed for identification of acid fast bacteria, yeast and fungi in all tissue specimens.

Immunohistochemical labeling for molecules of relevance to the diagnosis of BL was performed on 4-µm paraffin sections after antigen heat retrieval in 50 mM Tris/2 mM EDTA buffer solution, pH 9.0, for 30 minutes in a microwave oven. The following markers were analyzed: CD20 (clone L26, dilution 1:100; DAKO, Glostrup, Denmark), Ki-67 (MIB-1, dilution 1:100; DAKO), CD10 (56C6, dilution 1:20, Novocastra, Newcastle upon Tyne, United Kingdom), BCL2 (124, dilution 1:25, DAKO), BCL6 (PG-B6p, dilution 1:20, DAKO), and LMP1 (CS1-4, dilution 1:20, DAKO). Immunostaining was performed in an automated Ventana NeXes staining machine (Ventana Medical System, Inc., Tucson, AZ) according to the manufacturers' instructions.

The detection of EBER1-2 mRNAs was carried out by in situ hybridization on 4-µm paraffin tissue sections using a fluorescein-conjugated EBV (EBER) peptide nucleic acid probe (DAKO). The reaction was visualized with alkaline phosphatase conjugated anti-FITC sheep IgG Fab fragments (Roche, Mannheim, Germany) followed by 5-bromo-4-chloro-3-indolyl-phosphatase, 4-nitrobluetetrazolium (Roche) and MgCl2 incubation.

Fluorescence in situ hybridization (FISH) for detection of the breakpoints in *MYC* locus was performed on routine paraffin sections as described previously.[12] Two flanking probe sets were selected to detect most of the breakpoints reported in both endemic and nonendemic BL and designed for FISH segregation assay. Their selection and detailed characterization are described elsewhere (Haralambieva et al, in press).

## Case reports

### *Case No. 1*

A 39-year-old Dutch, HIV-negative and previously healthy male patient presented with right axillar lymphadenopathy. A fine-needle aspiration cytology of the enlarged lymph node was interpreted as reactive due to the extensive presence of histiocytes. However, flow cytometric analysis of the aspirate suggested a clonal B-cell proliferation. Histologic examination of a removed lymph node indicated BL combined with epithelioid cell granulomas. Since the extensive epithelioid granulomatous reaction caused difficulties in interpretation, the case was referred for consultation where the diagnosis of BL was confirmed. The patient had no B symptoms. Computed tomography of the abdomen revealed an infiltration of the small intestine by the malignancy, but no other sites of involvement were detected by the routine staging procedure (stage IIIE). The patient was treated with intensive chemotherapy according to the HOVON 27 protocol, consisting of doxorubicin, cyclophosphamide, Uromitexan, and prednisone, followed by a second course of prednisone, etoposide, and mitoxantrone and autologous stem cell transplantation. The patient received also central

nervous system prophylaxis with intrathecal application of methotrexate and prednisone. He remained in a complete remission without evidence of active disease (1-year follow-up).

***Case No. 2***

A 52-year-old and previously healthy male patient presented with a swelling in the left submandibular area. No HIV test was performed. Physical examination indicated a lymph node with a diameter of 2 cm. Histologic examination showed partial involvement by BL together with an extensive granulomatous reaction. The sample was referred for consultation, and the diagnosis of BL was confirmed. The staging procedure revealed no other localization of the tumor and no B symptoms were reported (stage IA). The patient was treated with standard chemotherapy (CHOP) and radiotherapy of the involved area. Since then he remained in complete remission (1-year follow-up).

***Case No. 3***

An 8-year-old, previously healthy Dutch girl presented with severe abdominal pain. The ultrasound and computed tomography procedures showed bulky mesenteric and mediastinal masses. A surgical sample from the mesenteric tumor showed morphologic and immunophenotypic features of BL. In the periphery of the sample, florid aggregates of epithelioid histiocytes were noted. The HIV status of the patient was not assessed. Serological investigations showed for EBV the following pattern: IgM anti-viral capside antigen < 32, IgG antiviral capside antigen >10240, IgG anti early antigen (EA) >256, IgG anti Nuclear antigen >150. The patient was diagnosed as BL stage III and was treated with an intensive chemotherapy regimen according to the NHL-94 Dutch Childhood Oncology Group protocol, consisting of induction by vincristine, cyclophosphamide, prednisone, and intrathecal polychemotherapy followed by maintenance polychemotherapy consisting of vincristine, cyclophosphamide, adriamycin, high-dose methotrexate, prednisone and intrathecal polychemotherapy. After chemotherapy the patient remained in complete remission (8-year follow-up).

***Case No. 4***

A 4-year-old, previously healthy and HIV-negative, Dutch boy presented in the emergency unit with episodes of severe abdominal pain. An ileocecal invagination was diagnosed and initially treated conservatively. However, an ultrasound examination revealed an ileocecal mass of 5 × 3 × 3 cm, and the patient underwent an explorative laparotomy. Microscopic examination of the specimen revealed infiltration of the intestinal wall by BL. At the periphery of the tumor and also admixed with the tumor cells, a prominent epithelioid histiocytic reaction was noted. The examined lymph nodes showed florid follicular hyperplasia and focal lymphoma involvement again accompanied by the epithelioid histiocytic reaction. A staging procedure revealed stage II and the patient was treated according to the LMB-96 protocol with vincristine, yclophosphamide, adriamycin, and prednisone. After 2 courses of chemotherapy, complete remission was achieved and the patient showed no evidence of disease after completion of chemotherapy (3-year follow-up).

**Results**

The clinical data and the results of immunostaining, EBER in situ hybridization, and FISH are presented in Table 1.

In all four samples, histologic examination revealed total or partial involvement of tumor composed of small- to medium sized cells with round nuclei and 2 to 5 non prominent nucleoli (Fig.1A). Numerous mitotic figures and apoptotic bodies accounted for the high proliferation rate of the tumor. A variable number of tingible body macrophages was admixed with the neoplastic cells giving rise to a starry sky appearance. In all the samples, there was a prominent infiltration of epithelioid histiocytes, forming small clusters and granulomas of different size. In 3 of 4 cases, they were intermingled with the tumor, replacing its usual monomorphic appearance (Fig. 1B, C). Occasionally, granulomas did also spread in the reactive areas of the samples. The granulomas showed no central necrosis (Fig. 1C), although in case no. 4 they were closely related to the small area of tumor necrosis. In case nos. 2 and 4, occasional giant cells were identified within the granulomas (Fig. 1D).

**TABLE 1.Main Clinical and Pathological Findings**

| patient | age | site | HIV status | stage | histochemistry | immunohistochemistry | EBER | FISH myc | Outcome |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 39 | right axillar lymph node, small intestine | - | IIIE | ZN – no acid fast bacteria PAS, Grocott – no microorg | CD20+, CD3-, CD5-, CD79a+, CD10+, BCL2-, BCL6+, Ki67:95% | + | + | Complete remission (1year follow up) |
| 2 | 52 | submandibular lymph node | Not tested | IA | ZN – no acid fast bacteria PAS, Grocott – no microorg | CD20+, CD3-, CD5-, CD79a+, CD10+, BCL2-, BCL6+, Ki67:99% | + | + | Complete remission (1year follow up) |
| 3 | 8 | mesenteric tumor | Not tested | IVB | ZN – no acid fast bacteria PAS, Grocott – no microorg | CD20+, CD3-, CD10+, BCL2-, BCL6+, IgM+/kappa+, Ki67:98% | + | + | Complete remission (8 years follow up) |
| 4 | 4 | Ileocaecal tumor | - | IIB | ZN – no acid fast bacteria PAS, Grocott – no microorg | CD20+, CD3-, CD10+, BCL2-, BCL6+ IgM/IgD+/kappa+, Ki67:99%, | + | + | Complete remission (3 years follow up) |

In all 4 cases, the Ziehl-Neelsen, periodic acid-Schiff and Grocott stainings did not reveal any acid-fast bacteria, yeasts, or fungi. In addition, PCR performed on DNA preparations from case nos. 1 to 3 did not identify *Mycobacterium tuberculosis* (data not shown). Furthermore, no Schaumann crystals, asteroid bodies, or foreign bodies were observed within the granulomas.

In 3 cases, the pronounced granulomatous reaction caused difficulties in interpretation and delay in diagnosis. Two of the samples were referred for consultation, and the aspiration cytology was false negative in one of them. In all cases, immunohistochemistry confirmed the presence of a highly proliferative B cell tumor (Ki67 index >95%) of germinal center phenotype (CD10 and BCL6 positive, BCL2 negative; Table 1). In all four samples, EBER in situ hybridization was positive for the tumor cells indicating EBV infection (Fig. 1E). All samples lacked expression of LMP1, indicating a latency I phenotype. In addition, in all four samples, a translocation breakpoint in the region of 50-kb 3' to 170-kb 5' of *MYC*, including *MYC* itself was identified by segregation FISH (Haralambieva et al, in press).

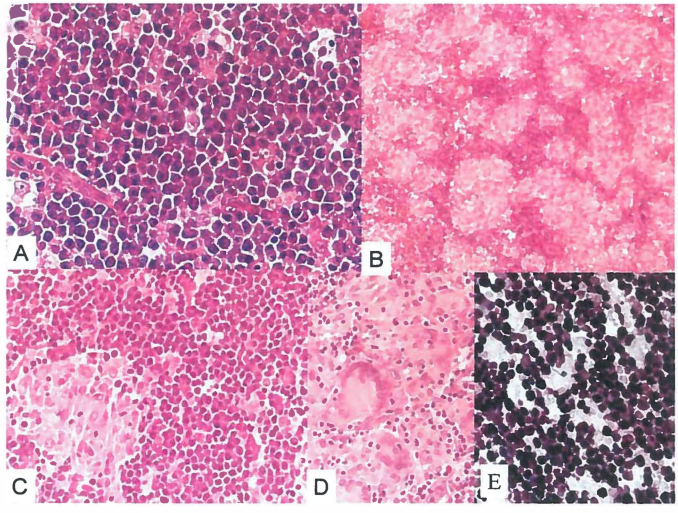


**FIGURE 1.** A composite picture, representing the details of the four described cases. **A**: Classic picture of BL, composed of small to medium size cells with round nuclei and scant cytoplasm. **B**: Numerous granulomas that obscure the presence of BL lymphoma. **C**: A small granuloma composed of epithelioid histiocytes, lacking central necrosis; note the Burkitt lymphoma at the right site of the picture. **D**: An occasional giant cell that may be found in the granulomas. **E**: All four BL cases are strongly positive for EBER1-2 m RNA by in situ hybridization.

**Discussion**

Here we report on an unusual combination of BL with an extensive epithelioid cell granulomatous reaction. Although well recognized in T-cell NHL and Hodgkin disease,[1,7,8,17,20,21] this phenomenon has been rarely reported in B-cell lymphomas.[3,9,11,13,15,23] We observed this phenomenon in four cases that were retrieved from the revision of a series of 67 BL from Europe, South America, and equatorial Africa (Haralambieva at al, in press), but we think that this phenomenon is overrepresented in our series since 2 of the 4 cases were sent to us for consultation and a third case was sent because the referring pathologist did know that we were interested in this particular phenomenon.

Previously, Halls et al [11] reported on 2 cases of BL with extensive necrosis surrounded by epithelioid cell granulomas mimicking tuberculosis. In contrast, necrotic areas were found in only one sample of our study and the granulomas did not contain central necrosis. A similar phenomenon has been described in seven patients with a small noncleaved lymphoma,[13] and probably at least part of them represented BL. In keeping with these two reports, the presence of a florid granulomatous reaction in our samples led to difficulties in the cytologic and histopathologic interpretation. In addition, Hollingsworth et al described an important clinical implication of this phenomenon, correlating the presence of the granulomas to a favourable clinical outcome of the patients.[13] All patients in our series achieved a complete remission and remained without evidence of disease; however, the follow-up period for especially the adult patients is too short to draw any firm conclusions. The agents that provoke a granulomatous reaction in malignant lymphomas remain unclear. Hall et al [11] suggested that the extensive tumor necrosis itself leads to the granulomatous response. Indeed, BL is a tumor with an especially high rate of proliferation and apoptosis; however, in our cases, we did not find large necrotic areas as reported by Hall et al.[11] Moreover, necrosis as observed in lymph node infarcts due to non-Hodgkin lymphoma involvement are often demarcated by a fibrohistiocytic wall but generally does not evoke a granulomatous reaction. In addition, necrosis can not explain the presence of a granulomatous reaction in other tumors nor its presence at distant uninvolved sites as can occur in Hodgkin lymphoma.

A granulomatous reaction may also be caused by a concomitant infection by tuberculosis, yeast, fungi, or other microorganisms, or by the presence of inert foreign bodies including fecal contents in cases of intestinal perforation, but it might also occur coincidentally without identification of any etiologic agent. In all 4 cases, we tried to exclude the involvement of any agent at the local tissue level by histochemical stainings (all cases) as well as PCR for mycobacteria (3 cases).

Furthermore, 2 patients tested were HIV-negative, and none of the patients had clinical signs of an underlying immunodeficiency, which was supported by the uneventful outcome after the very intensive chemotherapy.

Our observation that all four BL cases were EBV associated while originating from a nonendemic area suggests that EBV may have been a causative factor. Our 4 cases were retrieved from a series of 42 BL from the Netherlands, with EBV being tested in 35 of the 42 cases. Excluding the current 4 (index) cases, only 8 of 31 BL (26%) were EBV positive; therefore, we think that this finding is not coincidental (the association between EBV and granulomas was highly significant; $\chi 2$ test and Fisher exact test; $P < 0.05$). However, the current series of BL with a granulomatous reaction is very small, and studies on the EBV status in a larger series of lymphomas with a granulomatous reaction, including the already reported cases,[11,13] might clarify this issue.

What could be the pathogenesis of this granulomatous reaction in EBV-positive BL, and what could explain why only very few EBV-positive BL, including none of the 18 EBV positive BL cases from equatorial Africa and South America (Haralambieva et al, submitted for publication), showed this phenomenon? EBV nuclear antigen 1 (EBNA1) is the viral protein consistently expressed in all EBV-associated malignancies and in fact is the only EBV antigen expressed in BL (latency I phenotype). Whereas other EBV antigens like EBNA3A, B, and C are major targets of cytotoxic T lymphocytes, EBNA1 does not appear to be so.[10,17,18] Recent studies [2,10,19] suggest that CD4+ T helper cells are capable of recognizing EBNA1 in an HLA class II restricted way and to develop a $T_H1$ immune response, linked to delayed type hypersensitivity reaction and formation of granulomas. Interferon-$\gamma$, a $T_H1$ cluster cytokine, is a powerful activator of macrophages and the most important factor in transforming macrophages into epithelioid and multinucleated giant cells. Interestingly, various CD4+ epitopes within the EBNA1 protein are linked to different HLA class II restricting alleles [2,10,16,19]; therefore, the presence and magnitude of the $T_H1$ response and concomitant production of granuloma inducing cytokines may vary dependent on the HLA polymorphism. Thus, this restriction to certain HLA alleles might explain why such a florid granulomatous reaction is only seen in very few individuals. Finally, it might be hypothesized that this EBV specific $T_H1$ response is also correlated to the relatively favorable outcome of these BL patients.

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# Chapter 7

## Multiple myeloma and chromosomal translocations

Multiple myeloma is a bone-marrow based, multifocal neoplasm derived from a clonal expansion of bone marrow plasma cells, representing the immunoglobulin secreting end-stage B-cells. The disease spans a spectrum of clinical conditions ranging from asymptotic smoldering and indolent forms to aggressive disease with plasma cell infiltration of various organs, severe osteolytic lesions or osteoporosis with multiple bone fractures, plasma cell leukemia or abnormal immunoglobulin tissue deposition,.[1,2,3] The presence of a monoclonal (-M) protein without any further evidences of multiple myeloma or other related disorder is categorized as a monoclonal gammopathy of undetermined significance (MGUS). The disorder is considered to be benign since the clone is stable and the majority of the patients remain asymptomatic, however, after many years approximately 25% of them still develop multiple myeloma, light chain deposition disease (LCDD), macroglobulinemia or an other lymphoproliferative disorder.[1,3,4]

Recent studies emphasized the importance of genetic abnormalities regarding the biology and clinical outcome of MM patients.[5,6,7,8,9] More than one-half of MM patients carry translocations involving the IgH locus and the remaining patients have hyperdiploidy as the hallmark of their disease.[2,9,10] Other essential genetic aberrations represent chromosome 13 abnormalities, Ras mutations or inactivation of p53 by either deletion or mutation. [9,11,12,13]

**Table 1 Prevalence and clinical importance of IgH translocations**

| Abnormality | MGUS&SMM (%) | MM (%) | Oncogenes | Effect on prognosis |
|---|---|---|---|---|
| IgL translocation | <20 | <20 | c-*myc* and others | Unknown |
| IgH translocations | 35-50 | 50-70 | | |
| t(4;14)(p16.3;q32) | 2-10 | 15 | *FGFR3* and *MMSET* | Adverse |
| t(11;14)(q13;q32) | 15-30 | 16 | *cyclin D1* and *MYEOV* | Favorable |
| t(14;16)(q23;q23) | 2-5 | 5 | c-maf | Adverse |
| t(6;14)(p21;q32) | Unknown | 4 | *cyclin D3*, other | Unknown |
| Other IgH | 10-15 | 15-30 | *mafB*, *MUM1*, others | Unknown |

MGUS – monoclonal gammopathy of undetermined significance; MM – multiple myeloma; SMM – smoldering MM

Interestingly, almost all genetic lesions identified in MM are also present in MGUS [14, 15] and the incidence of IgH translocation in MGUS is nearly as high as in MM, indicating that they represent an early genetic event. FISH studies demonstrate that IgH translocations are detectable in nearly 50% of MGUS and SMM, 55-70% of MM, 80% of primary PC leukemia and > 90% of extramedullary plasmacytomas and human myeloma cell lines (HMCL) [2,5,6,9]. As HMCL are usually generated from the more aggressive MM stages they may not precisely reflect the incidence of abnormalities found *in vivo* in the tumors but rather represent an ultimate stage of tumor progression.[16] Limited data on translocations involving the light chain genes (IgL) indicate the prevalence of IgL –λ translocations in ~ 10% in MGUS/SMM and ~20% in advanced MM and HMCL. IgL-κ translocations are relatively infrequent [3,5,6,9] In contrast to the other B-cell neoplasias, MM show a marked diversity of the chromosomal partners involved in Ig translocations (Table 1). Apart from *MYC* at 8q24, there are four major IgH translocation partners 11q13/ *cyclin D1*, 4p16/*FGFR3*/ *MMSET*, 6p21/*cyclin D3* and 16q23/*c-maf*, with the latter two also involved in Igλ translocation [1, 3,17-24]. Other recurrent translocations that occur less frequently involve 20q11/*maf-B* and 6p25/*IRF-4/MUM-1*.[25,26] It has been proposed that most primary IgH translocations result from errors in B-cell specific DNA remodeling mechanisms: mostly the *IgH* switch recombination and less often the somatic hypermutation machinery. [5,27] Since the translocation breakpoints are located within or very near *IgH* switch regions, *IgH* enhancers segregate into both derivative chromosomes and in consequence one or two potential oncogenes on each derivative chromosome can be deregulated. Thus, the t(4;14) results in deregulation in *FGFR3* on der14 and *MMSET* on der4.[21] Similarly, concurrent up-regulation of *cyclin D1* and another putative 11q13 oncogene, *MYEOV* has been reported in MM cell lines that carry t(11;14).[28]

Of note, the influence of IgH enhancers may extend over a large genomic region as is the case for c–maf in the KMS-11 cell line, where the gene is located at ~1Mb from the enhancer.[23] In such cases oncogenes located hundreds of kilobases away can still be transcriptionally up-regulated. In parallel with many other translocations, IgH translocations in MM are often unbalanced. For instance the t(4;14) is unbalanced in ~25% of MM patients , however, these tumors never show loss of der 4 but always of der(14) involving *FGFR3*, indicating that *MMSET* and not *FGFR3* is the target gene of the translocation. This is further supported by the observation that some MM cases show retention of der(14) but nevertheless loss of *FGFR3* expression. [29]

Translocations that involve *MYC* represent a paradigm for secondary translocations in MM and their incidence is related to disease progression from ~3% in MGUS/SMM, 15% in MM and > 90% of HMCL, with marked heterogeneity within the tumor cell population [5,30,31,32,33]. Most karyotypic abnormalities (not necessarily involving IgH loci) are complex translocations and insertions that are unbalanced and frequently involve more than two chromosomes. They also include amplification, duplication and inversion.[31,32] Simple reciprocal translocations involving *MYC* and the Ig loci are less frequent and are usually not mediated by somatic hypermutation and/or class switch recombination.

Current data suggest that specific cytogenetic abnormalities identify subsets of MM patients with unique clinical features and therapeutic response [34-38]. The t(4;14) is an unfavorable prognostic factor in MM patients treated either with conventional or high dose chemotherapy. No difference between patients with or without FGFR3 expression have been found, again indicating that *MMSET* is the target gene of this translocation.[35,39] This translocation is associated with more aggressive clinical features but also with an IgA isotype and λ-light chain use.[6,35] Limited data indicate that the t(14;16) is associated with similar poor clinical outcome.[6] Of note, there is a high prevalence of complete or partial deletion of a long arm of the chromosome 13q or monosomy 13 (Δ13) among patients with t(4;14) and t(14;16) and Δ13 on its own already represents an adverse prognostic factor, both in patients treated with conventional and high dose chemotherapy.[37,40,41] Since both genetic aberrations are seen at very early stages of the disease, and the order of these events is unknown, it could be discussed which lesion, the translocation or Δ13 is most relevant. The high prevalence of Δ13 in patients with t(4;14) and t(14;16) suggests primacy for Δ13, however it is also possible that these translocations allow Δ13 to be "tolerated" by the tumor cells.

The data on the prognostic value of t(11;14) in MM tumors are controversial, however, most recent studies indicate that MM patients with t(11;14) have a marginally better survival following conventional chemotherapy.[18,42] Of note, the outcome significantly improves in patients treated with high dose chemotherapy and stem cell support. [35,43] The t(11;14) is associated with an oligosecretory and non-secretory variant, CD20 expression and lymphoplasmocytic morphology. Some cases express IgM instead of IgG or IgA [42,44,45]

There are limited data available on MM patients that carry t(6;14)(p21;q32) and t(6;14)(p25;q32), therefore the clinical implication and prognostic significance of these translocations remain enigmatic. The clinical and prognostic implications of *MYC* abnormalities are also not known, but likely to be negative related to overall survival, since *MYC* aberrations are more frequent in advanced tumors with a high proliferative index.

Genetic lesions in MM have been studied using various methodologies, including conventional cytogenetics, metaphase spectral karyotype imaging/multicolor-FISH (SKY/M-FISH), Comparative-Genomic-Hybridization (CGH), FISH, FICTION and microarray gene expression analysis. Cytogenetic analysis is only feasible in advanced MM and plasma cell leukemia. Due to the initially low tumor load and the low proliferation rate of the tumor, many cases with less advanced disease (50-70%) reveal normal karyotypes that originate from the myeloid elements instead of tumor cells. [34,38,46,47]. Even among patients with abnormal karyotypes, some of the most important aberrations may be cryptic.[20,23] For instance the t(4;14)(p16.3;q32) occurs in 15-20% of all MM but can not be recognized in MM karyotypes.[20,21,22] Similarly the t(14;16)(q32;q23) is difficult to detect by conventional G-banding, while the detection success rate significantly improves by the usage of SKY/M-FISH.[23,48,49,50] In contrast, karyotypic analysis is a much more sensitive method for the detection of numerical chromosomal abnormalities. Multicolor metaphase FISH (M-FISH/SKY) is applied to improve the accuracy of conventional cytogenetics, especially for analysis of complex karyotypes.[48,49,50] FISH represents a preferable technology for screening for various (already established) genetic aberrations since it can be performed on non-dividing cells and has a high sensitivity and specificity.[10,11,14,15,40,41,42] It, therefore, has been successfully used for recognition of numerical aberrations (trisomies/monosomies), deletions such as del13q and del17p13.1 but especially of translocations involving the IgH , IgL and other loci. To improve the detection of a given genetic aberration, especially in patients with a low tumor burden, FISH is preferably combined with cell purification procedures (e.g. CD138+ microbead selection) or simultaneous immunofluorescence. Translocations are successfully detected using segregation FISH, co-localization FISH or a combination of both techniques. Specific problems as $V_H$ rearrangement with deletion of DNA template that results in a weak FISH signal and the unbalanced nature of some MM translocations should be considered in the evaluation of FISH results. The t(4;14)(p16.3;q32) can be also detected by reverse transcription-PCR detection of the *IgH-MMSET* hybrid transcript.[20,21]

Gene expression profiling is also used for the molecular classification of MM and its relation to MGUS.[51,52] Recent studies suggest that the majority of the recurrent IgH translocations are also detectable by gene expression profiling with spiked expression patterns of the genes involved in the specific translocations.[51]

Many recent reports emphasize the importance of cyclin D genes in the pathogenesis and progression of MM and related plasma cell disorders, [17,18, 22, 35-37, 42-45,51, 53-57] ,however, there are still many open questions. For instance, in mantle cell lymphoma the presence of t(11;14) strongly correlates with cyclin D1 RNA and protein over-expression [58] as a functional endpoint of the genetic lesion. In contrast, in

MM cyclin D1 expression and 11q13 aberrations are not that strictly correlated in MM. Thus up to 30% of MM patients show Cyclin D1 overexpression as measured by immunohistochemistry or RT-PCR, while the incidence of the t(11;14) translocation is 15-20% as assessed by FISH studies. Although the discrepancy may be partially due to methodological differences and/or patient's selection, it suggests that mechanisms other than an 11q13 breakpoint may trigger cyclin D1 overexpression in MM. In chapter 8 we investigated this issue by studying a large series of MM with various methods.

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## Chapter 8

# Different mechanisms of cyclin D1 overexpression in multiple myeloma revealed by fluorescence in situ hybridization and quantitative analysis of mRNAlevels

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The t(11;14)(q13;q32) is the most common translocation in multiple myeloma (MM), resulting in up-regulation of cyclin D1. We used a segregation fluorescence in situ hybridization (FISH) assay to detect t(11;14) breakpoints in primary MM cases and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) to quantify cyclin D1 and MYEOV (myeloma overexpressed) expression, another putative oncogene located on chromosome 11q13. High levels of cyclin D1 mRNA (cyclin D1/TBP [TATA box binding protein] ratio > 95) were found exclusively in the presence of a t(11;14) translocation (11/48 cases; $P <$ .00001). In addition, a subgroup of MM cases (15/48) with intermediate to low cyclin D1 mRNA (cyclin D1/TBP ratio between 2.3 and 20) was identified. FISH analysis ruled out a t(11;14) translocation and 11q13 amplification in these cases; however, in 13 of 15 patients a chromosome 11 polysomy was demonstrated ($P <$ .0001). These results indicate an effect of gene dosage as an alternative mechanism of cyclin D1 deregulation in MM. The absence of chromosome 11 abnormalities in 2 of 15 patients with intermediate cyclin D1 expression supports that there are presumably other mechanism(s) of cyclin D1 deregulation in MM patients. Our data indicate that deregulation of MYEOV is not favored in MM and further strengthens the role of cyclin D1 overexpression in lymphoid malignancies with a t(11;14)(q13;q32) translocation.

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## Introduction

Translocations involving the immunoglobulin heavy chain region (IgH) on chromosome 14q32 are an important cytogenetic event in the pathogenesis of various B-cell lymphoid neoplasms such as multiple myeloma (MM), mantle cell lymphoma (MCL), Burkitt lymphoma, and follicular lymphoma.[1,2] In MM, these translocations are thought to be an early event in the pathogenesis, occurring mostly during class switch recombination in terminally differentiated B cells and leading to deregulation of translocated genes under the influence of the IgH enhancers on 14q32. The IgH locus contains at least 3 different enhancers: the 5' intronic μ enhancer (Eμ) located in the intron between the IgH joining (JH) and switch μ (Sμ) sequences and two 3'-IgH enhancers located downstream of the constant region genes (Eα1 and Eα2).[3,4] The breakpoints in the IgH locus are usually inside switch regions and can result in separating and splitting of 2 different enhancers to each derivative chromosome, theoretically leading to deregulation of genes on both derivative chromosomes. Occasionally, breaks also occur in the JH region or in the diversity and joining (DJ) region on the basis of hypermutations followed by chromosomal breakage. More than 20 different chromosomal partner regions that translocate to 14q32 have been identified so far in MM,[5-8] of which the t(11;14)(q13;q32) is the most common translocation with a reported frequency of 15% to 20% based on fluorescence in situ hybridization (FISH) or conventional cytogenetic analysis.[8-12] The t(11;14)(q13;q32) translocation results in up-regulation of the cell cycle regulatory proto-oncogene *CCND1/cyclin D1,* which is normally not expressed in plasma cells. In MM the breakpoints are scattered within a 360-kb region between *CCND1* and *MYEOV*.[13-15] *MYEOV* is a putative oncogene previously identified by the application of a NIH3T3 tumorigenicity assay with DNA from a gastric carcinoma1[5] and lacks homology to any known gene. *MYEOV* is located 360 kb centromeric of *CCND1* and its activation, concurrent to *CCND1* by juxtaposition of *MYEOV* to either the 5' Eμ or the 3' Eα IgH enhancer, has recently been described.[15] However, although MYEOV (myeloma overexpressed) was overexpressed in a subset of MM cell lines with t(11;14), it has never been investigated in primary MM cases. The aim of this study was, therefore, to investigate both cyclin D1 and MYEOV deregulation by quantitative reverse transcriptase–polymerase chain reaction (QRT-PCR) in a series of primary MM cases and to explore its relationship to the presence of a t(11;14)(q13;q32) translocation, as assessed by a FISH assay.[16]

## Patients, materials, and methods

### *Tissue samples*

Sixty formalin-fixed, paraffin-embedded blocks of 48 patients with MM diagnosed between 1991 and 2002 were retrieved from the files of the Institute of Pathology, Technical University of Munich (Munich, Germany).

In 9 of these MM cases, 2 or 3 biopsies taken at different time points over a period of up to 5 years were available. The biopsies were not decalcified, as most of the material was obtained during surgery from large osteolytic lesions. The cases were graded according to the histologic grading criteria described by Bartl et al17 and were reviewed by 3 of the authors (L.Q.-M., M.K., and F.F.). Some of the cases have been reported previously as part of another study.[18] In addition, 14 formalin-fixed, paraffin-embedded blocks of non neoplastic lymphoid tissues (6 lymph nodes, 4 tonsils, and 4 decalcified bone marrow biopsies with reactive changes) were included as normal tissue controls.

### *Cell lines*

In addition to the primary cases, 7 human myeloma cell lines (KMM1, OPM2, U266, KMS5, KMS11, KMS12, and KMS18), 2 mantle cell lymphoma cell lines (Granta 519 and NCEB), one anaplastic large cell lymphoma cell line (Ki-JK), 2 diffuse large cell lymphoma cell lines (SUDHL-4 and SUDHL-10), and 4 carcinoma cell lines (HT29, A431, MDA-MB231, and MDA-B468) were used in the study. The carcinoma cell lines were acquired from American Type Culture Collection (ATCC; Manassas, VA) and cultured as recommended by the supplier. All lymphoid cell lines were cultured in RPMI 1640 medium supplemented with 15% fetal calf serum, 2 mM glutamine, 10 U/mL penicillin, and 60 μg/mL streptomycin (Invitrogen, Life Technologies, Karlsruhe, Germany).

### *RNA extraction and QRT-PCR*

Tissue preparation, microdissection of pure tumor cell populations, and RNA extraction from formalin-fixed tissues were performed as described previously.[19] Briefly, approximately 10 000 tumor cells were microdissected from each deparaffinized sample. Cells were lysed in 200 μL Tris/EDTA (tris(hydroxymethyl)aminomethane/ethylenediaminetetraacetic acid) lysis buffer and 500 μg/mL proteinase K. After complete digestion at 60°C, RNA was purified by phenol-chloroform extraction, precipitated, and

dissolved in 20 µL H2O. Total RNA from cell lines was isolated using the acid-phenol-chloroform guanidinium method.[20] Extracted RNA was DNase treated by incubating the RNA for 1 hour at 37°C with 10U of DNAse I (Roche, Basel, Switzerland). Reverse transcription was performed with 250 ng of random hexamers (Roche) and 200U of Superscript II–reverse transcriptase (Invitrogen, Life Technologies) in a final volume of 20 µL, following the manufacturer's directions.

QRT-PCR analyses were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

MYEOV primers and probe were as follows, yielding a 80-bp product for MYEOV (designated by the nucleotide position relative to *MYEOV* GenBank accession number AJ223366): *MYEOV*-901 sense, 5'-CGGTGAGAGGAGCATTTGTGT-3'; *MYEOV*-980 antisense, 5'-GGCACCCTTGTCTCCCTTGT-3'; and *MYEOV*-930 probe, 5'-FAM TTTGCTGCTGGAGCTGGTGACCG-3'TAMRA. PCR reaction conditions, sequences for primers and probes for cyclin D1 and TBP (TATA box binding protein) as housekeeping gene control, and quantification procedures have been published.[19] The cutoff value for altered cyclin D1 expression was based on the mean value of cyclin D1/TBP ratios determined in 8 reactive lymphoid tissues and bone marrow samples + 5 standard deviations and was 2.33 (mean, 0.84; range, 0.31-1.38). Ratios between 2.33 and 20 were arbitrarily considered to represent low (2.33-9.9) to intermediate (10-20) cyclin D1 overexpression and ratios above 20 or more to represent high cyclin D1 overexpression. Amounts of MYEOV and TBP RNA were calculated using linear regression analysis from an external standard curve generated from KMS12 RNA. MYEOV/TBP ratios were determined in 14 reactive tissues from lymph nodes (LNs), bone marrow (BM), and tonsils (mean, 0.15; range, 0.02-0.5). MYEOV/TBP ratios below 1.0 (mean + 5 standard deviations) were arbitrarily considered to represent normal or negative MYEOV levels.

***FISH***

B-cell leukemia/lymphoma-1 (BCL-1) breakpoints (5' and 3'of the *CCND1* gene) were detected by a previously designed interphase FISH segregation assay.[13,21] In brief, 2 probe sets were selected after mapping of large contigs of probes on normal DNA fibers resulting in a so-called color barcode of the BCL-1/11q13 region. Subsequently, a consecutive mapping of all possible breakpoints by applying the same set of probes on DNA fibers from tumor material with a putative BCL-1/11q13 breakpoint was performed. A schematic representation of the probe sets for detection of the BCL-1/11q13 breakpoints is shown in Figure 1A. The pooled cosmids cos6.7/cCl-11-44 were used in combination with the pooled cos6.31/cosH1.5 to detect the breakpoints within an 800-kb region around *CCND1*. In approximately 8% of MCL cases, we previously identified a mono-allelic breakpoint both immediately 3' as well as 5' of the *CCND1* gene.[24] Because these mono-allelic double breaks will not be detected with the 800-kb flanking probe set, MM cases negative for this latter probe set were studied with cos6.22/*CCND1* in combination with the pooled cos6.31 and cosH1.5 (Figure 1A).

To determine the position of *CCND1* relative to IgH enhancers (Figure 1B), the breakpoint-positive cases were analyzed by a colocalization assay using 2 probe sets (Figure 1A-B): (1) cos6.22 (covering *CCND1*) in combination with the pooled cosmid U2-2 (covering the JH and part of the diversity and heavy region [DH] gene segments) and cos3/64 (covering the JH, Cδ, and Cµ gene segments) as described by Vaandrager et al [23] and Matsuda et al [22]; and (2) cos6.22/*CCND1* in combination with PAC27M16 located immediately 3' of the immunoglobulin Cα2 region (provided by Dr D. Cox, Research Institute, Hospital for Sick Children, Toronto, ON, Canada).

Probes were labeled by the Kreatech Universal Linkage System (ULS) nonenzymatic labeling method (Kreatech Diagnostics, Amsterdam, the Netherlands) with biotin-16-aUTP or digoxygenin-11-dUTP (Roche). The adapted conditions for the hybridization on routine tissue sections from formaldehyde-fixed, paraffin-embedded tissue blocks were described recently.[16]

Signals were considered colocalized when the distance between them was equal or smaller than the size of one hybridization signal. Any breakpoint should result in segregation of one or both probe sets and the presence of single red and/or single green hybridization signals in tissue sections.[16] In the 7 negative control tissues, single signals were detected in 1% to 7% (mean, 4.9%; standard deviation, 1.9%) of the nuclei and the cutoff levels for both probe sets were set at 11.0% (mean plus 3 times the standard deviation).

Samples that showed evidence of increased copy number for *CCND1* locus were studied separately using the CEP11 probe, labeled with SpectrumGreen, that hybridizes to the alpha satellite DNAsequence located at the centromere of chromosome 11 (11p11.1-q11) in combination with *CCND1* gene locus (11q13)–specific probe labeled with SpectrumOrange (Vysis, Downers Grove, United Kingdom). Tissue pretreatment was performed as previously described.[25] FISH signals of at least 100 nuclei of each tumor area were evaluated by 2 investigators using a laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany) equipped with epifluorescence optics (C-Apochromat X 63/1.2 Wcorr) and an appropriate combination of emission filters (BP-500-550 IR, BP-565-615 IR, LP 650).

Image processing was carried out with Zeiss software (AIM 3.0). Following the criteria of Hopman et al,[26] each case was classified as amplified for the *CCND1* gene locus if there were more than twice as many clustered red cyclin D1 signals as green centromere 11 signals (ratio > 2). A polysomy 11 was diagnosed if

more than 20% of all nuclei showed more than 2 nuclear red signals accompanied by the same number of nuclear green signals (ratio 1).

***Immunohistochemistry***

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical System, Tucson, AZ) according to previously published procedures.[18] The cyclin D1 antibody used in this study was from Novocastra (Newcastle, United Kingdom; clone P2D11F11) and CD20 was from Dako (Glostrup, Denmark; clone L26). Images were recorded using a Hitachi camera HW/C20 installed in a Zeiss Axioplan microscope with Intellicam software. Adobe PhotoShop (San Jose, CA) was used for image processing.

***Statistical analysis***

To characterize differences in the mRNA levels between the various MM cases, descriptive statistics was used. For continuous variables, the Wilcoxon rank sum test was used to test for differences between patients with the t(11;14)(q13;q32) translocation versus other patients. The Fisher exact/chi-square test was used for the count data. To correlate between polysomy and nonpolysomy cases, the Wilcoxon rank sum test was used. All the tests were performed using functions of ctest package of R libraries for statistical computing (www.r-project.org).

**Results**

***Quantitation of cyclin D1 mRNA in 48 primary cases of MM***

To establish and validate the system for real-time cyclin D1 expression analysis we first analyzed a series of well-characterized cell lines (Table 1). Increased expression of cyclin D1 correlated with both DNA amplification in 4 carcinoma cell lines and with the lymphoid cell lines carrying t(11;14). The results of cyclin D1mRNA expression levels in the primary MM tumors are summarized in Table 2. Cyclin D1 mRNA transcript levels are clustered in 3 different groups (Figure 2). Group 1 included 11 (23%) cases displaying high cyclin D1 mRNA levels (cyclin D1/TBP ratio _ 95; median, 171.3; range, 96.76-666.25). A second group included 15 (31%) cases with low to intermediate cyclin D1 mRNA levels (cyclin D1/TBP ratio between 2.3 and 20; median, 7.64; range, 2.5-18.72). In 2 patients (cases 12 and 17), follow-up biopsies taken up to 5 years apart showed similar results, indicating that the overexpression of cyclin D1 remains stable over time. Finally, the third group comprised the remaining 22 (46%) cases with normal or negative expression of cyclin D1 mRNA (cyclin D1/TBP ratio between 0 and 2; median, 0.6; range, 0.11-2.03).

**Table 1 Cyclin D1 and MYEOV gene expression levels in lymphoma and carcinoma cell lines**

| *Cell Line* | *Origin* | *Q-RT-PCR* | | *11q13 status* |
|---|---|---|---|---|
| | | *CyD1* | *MYEOV* | |
| KMM1 | Multiple Myeloma | absent | 0.05 | ND |
| OPM2 | Multiple Myeloma | absent | 0.05 | not amplified |
| KMS5 | Multiple Myeloma | 0.07 | 0.42 | ND |
| KMS11 | Multiple Myeloma | 0.2 | 2.09 | not amplified |
| KMS12 | Multiple Myeloma | 29 | 6.05 | t(11;14)(q13;q32) |
| KMS18 | Multiple Myeloma | absent | 8.33 | not amplified |
| U266 | Multiple Myeloma | 4.2 | 0.2 | Insertion 11q*, Disomy |
| Ki-JK | Anaplastic Large Cell Lymphoma | absent | 0.59 | ND |
| NCEB | Mantle Cell Lymphoma | 28.4 | 21.36 | t(11;14)(q13;q32) |
| Granta 519 | Mantle Cell Lymphoma | 79 | 0.3 | t(11;14)(q13;q32) |
| SUDHL-4 | DLCL | absent | 0.06 | ND |
| SUDHL-10 | DLCL | absent | 2.84 | ND |
| HT29 | Gastric Carcinoma | 8.8 | 36.18 | amplified |
| A431 | Squamous Carcinoma | 34.6 | 275 | amplified |
| MDA-MB231 | Breast Carcinoma | 7.73 | 27.5 | amplified |
| MDA-MB468 | Breast Carcinoma | 10.15 | 21.5 | amplified |

**Table 2 FISH and real-time RT-PCR analysis in 48 cases of MM**

| *Case* | *Age* | *Sex* | *MG* | *Histology* | *Segregation FISH* | *Locus-Specific FISH* | *Q-RT-PCR CyD1* | *Q-RT-PCR MYEOV* | *IHC CyD1* |
|---|---|---|---|---|---|---|---|---|---|
| **Group 1: t(11;14) Positive and High CyD1 mRNA and Protein** | | | | | | | | | |
| 1 | 64 | M | G,κ | I-LP | NF | ND | 666.25 | 1.99 | +++ |
| 2a* | 78 | F | G,κ | I | Pos | ND | 425 | 9.57 | +++ |
| 2b* | | | | | Pos, ↑copies | ND | 571 | ND | +++ |
| 3 | 58 | M | G,κ | I-LP | Pos | ND | 408.09 | 3.3 | +++ |
| 4 | 59 | M | *** | I-LP | Pos±, ↑copies | ND | 192.9 | 3.71 | +++ |
| 5 | 61 | F | κ | I | Pos, ↑copies | ND | 175.9 | 0.55 | +++ |
| 6 | 81 | M | λ | I-LP | Pos±, ↑copies | ND | 166.71 | 0.03 | +++ |
| 7 | 56 | F | G,κ | I | Pos±, ↑copies | ND | 163.51 | 2.71 | +++ |
| 8 | 59 | M | κ | I-LP | Pos, ↑copies | ND | 145.22 | 0.35 | +++ |
| 9 | 76 | F | λ | I | Pos | ND | 139.24 | 44.4 | +++ |
| 10 | 40 | M | G,κ | I | Pos±, ↑copies | ND | 103.85 | 1.98 | +++ |
| 11* | 76 | M | κ | I-LP | Pos | ND | 96.76 | 1.79 | +++ |
| **Group 2: t(11;14) Negative and Low to Intermediate CyD1 mRNA and Protein** | | | | | | | | | |
| 12a | 67 | M | G,κ | I-LP | Neg, ↑copies | Polysomy | 18.72 | 7.58 | ++ |
| 12b | | | | | Neg, ↑copies | Polysomy | 14.23 | ND | ++ |
| 12c | | | | | Neg, ↑copies | Polysomy | 17.07 | ND | + |
| 13 | 78 | M | *** | I | Neg | Disomy | 15.44 | ND | ++ |
| 14 | 69 | F | G,κ | I | Neg | Disomy | 15.34 | 24.28 | ++ |
| 15§ | 32 | M | G,κ | I | Neg, ↑copies | Polysomy | 15.36 | 31.59 | + |
| 16 | 66 | M | G,κ | I-LP | Neg, ↑copies | Polysomy | 12.42 | 1.76 | + |
| 17a | 59 | M | G,κ | I | ND | Polysomy | 10.49 | 7.25 | + |
| 17b | | | | | Neg, ↑copies | Polysomy | 5.27 | ND | + |
| 17c | | | | | Neg, ↑copies | Polysomy | 6.7 | ND | + |
| 18 | 74 | F | G,κ | I | Neg, ↑copies | Polysomy | 3.78 | 11.34 | ++ |
| 19 | 74 | F | G,κ | I | Neg, ↑copies | Polysomy | 7.79 | 10.42 | Neg[1] |
| 20 | 60 | F | G,λ | II | Neg | Polysomy | 7.64 | 1.59 | Neg |
| 21 | 61 | M | *** | I | Neg, ↑copies | Polysomy | 6.6 | 19.95 | Neg[1] |
| 22* | 56 | F | *** | I-LP | Neg, ↑copies | Polysomy | 4.14 | 13.94 | Neg |
| 23 | 60 | F | G,κ | I | Neg, ↑copies | Polysomy | 3.63 | 0.36 | Neg |
| 24 | 68 | M | *** | I | Neg, ↑copies | Polysomy | 3.28 | 2.34 | Neg[1] |
| 25* | 69 | F | G,κ | I | Neg | Polysomy | 2.56 | 3.57 | Neg |
| 26 | 73 | M | G,κ | I | Neg, ↑copies | Polysomy | 2.5 | 3.2 | Neg |
| **Group 3: t(11;14) and CyD1 mRNA and Protein Negative** | | | | | | | | | |
| 27 | 76 | M | A,λ | II | Neg | ND | 2.03 | 0.36 | Neg |
| 28 | 48 | F | λ | II | Neg | ND | 1.55 | 0.18 | Neg |
| 29 | 63 | F | G,κ | II | Neg | ND | 1.26 | NF | Neg |
| 30 | 79 | F | *** | I | Neg | ND | 1.23 | 2.13 | Neg |
| 31 | 47 | F | κ | I | Neg | ND | 1.0 | 4.23 | Neg |
| 32 | 71 | F | G,κ | I | Neg | ND | 0.87 | 6.6 | ND |
| 33 | 52 | M | G,λ | I | Neg | ND | 0.77 | Absent | Neg |
| 34 | 72 | F | *** | II | Neg | ND | 0.77 | 0.44 | ND |
| 35 | 75 | M | *** | II | Neg, ↑copies | Polysomy | 0.67 | 0.01 | Neg |
| 36 | 74 | F | κ | I | Neg | ND | 0.62 | 0.8 | Neg |
| 37 | 55 | F | λ | I | Neg | ND | 0.57 | 1.14 | Neg |
| 38* | 63 | F | A,λ | I | Neg | ND | 0.46 | 13.38 | Neg |
| 39a | 75 | F | κ | I | Neg | ND | 0.44 | ND | Neg |
| 39b | | | | | Neg | ND | 0.14 | 2.48 | Neg |
| 40 | 62 | F | M,λ | II | Neg | ND | 0.7 | 2.66 | Neg |
| 41 | 69 | M | G,λ | I | Neg | ND | 0.38 | ND | Neg |
| 42a | 64 | M | G,κ | II | Neg | ND | 0.37 | ND | Neg |
| 42b | | | | | Neg | ND | 0.82 | 3.42 | Neg |
| 42c | | | | | Neg | ND | 0.42 | ND | Neg |
| 43 | 54 | M | λ | I-LP | Neg | ND | 0.27 | 4.14 | Neg |
| 44§ | 64 | F | G,λ | I | Neg | ND | 0.26 | 6.8 | Neg |
| 45 | 63 | M | G,λ | I | Neg | ND | 0.26 | 6.24 | Neg |
| 46 | 64 | F | G,λ | I | Neg | ND | 0.2 | 5.8 | Neg |
| 47 | 76 | M | G,κ | II | Neg | ND | 0.13 | 1.28 | Neg |
| 48 | 57 | M | *** | I | Neg | ND | 0.11 | 0.21 | Neg |

MG: monoclonal gammopathy; LP: lymphoplasmocytoid morphology; NF not feasible; ND not done; * positive for CD20 in a proportion of tumor cells; *** data are not available; ± segregation FISH pattern indicative of a translocation with subsequent loss of the derivative chromosome containing MYEOV (see Figure 1D for a representative example); § multiple biopsies were analyzed;
IHC score: cyclin D1 +++ _80% tumor cells strongly positive, ++20-50% tumor cells positive, +10-

20% tumor cells positive; ¶ cases were considered negative, however, rare bona fide plasma cells were positive for cyclin D1

***FISH analysis in primary cases of MM***

Fifty-five routine paraffin samples of 48 MM patients were analyzed by segregation FISH for the presence of breakpoints in the *CCND1* locus (Table 2). Case 1 was not informative because of the consistently weak hybridization signal. Overall, 10 patients (21%) showed evidence of a *CCND1* breakpoint by the presence of high numbers of segregated FISH signals on tissue sections with the 5' probe set (group 1; Figure 1C-E). Further analysis revealed a specific FISH signal pattern in 4 of these patients (cases 4, 6, 7, and 10) consisting of 2 co-localized signals representing the normal allele and a single red signal (Figure 1E). This pattern and the lack of a single green signal indicated the presence of a mono-allelic translocation break in the *CCND1* locus with a loss of the derivative chromosome der(11) carrying *MYEOV* (Figure 1A,E).

No additional breakpoints were detected in any of the breakpoint negative cases tested by the 3' probe set.

Colocalization FISH demonstrated that in all evaluable cases in group 1, *BCL-1/CCND1* was juxtaposed to IgH sequences. In cases 2, 4, 7, and 10, the 6.22/*CCND1* probe colocalized with PAC27M16 but not with cosmids U2-2 and cos3/64, suggesting that the breakpoints were localized either in the gamma or alpha switch region of the IgH locus and, thus, *CCND1* is only juxtaposed to 3'-Eα enhancers and not to the intronic 5'-Eμ enhancer. In cases 5, 6, and 8, the *CCND1* signal colocalized with a smaller red signal derived from U2-2 and cos3/64 indicating a break at JH or Sμ.

Since this interphase FISH assay cannot discriminate between a break in JH or Sμ, we cannot conclude whether *CCND1* is juxtaposed to all 5'-Eμ and the 3'-Eα enhancers (in case of a break within the JH region) or only the 3'-Eα enhancers (in case of a Sμ break). In cases 9 and 11, the 6.22/*CCND1* probe colocalized with PAC27M16, but all signals from cosmids U2-2 and cos3/64 were lost in tumor population although they were present in admixed non-MM cells. Consequently, we could not draw any conclusion on the exact breakpoint within the IgH locus. Case 3 was not analyzed by colocalization FISH because of lack of material.

In 22 samples of 19 patients, segregation FISH indicated the presence of an increased copy number for the*CCND1*locus (Figure 1F).

In order to confirm this finding and to be able to distinguish between low-level amplification from increased chromosome copy numbers (polysomy), these samples and all cases with intermediately elevated cyclin D1 levels (cases 12-26 and 35) were further analyzed by FISH with a *CCND1* locus–specific probe and a chromosome 11 centromeric probe. The results are listed in Table 2. Fourteen cases were scored as having an 11q13 polysomy (Figure 1G-H), while only 2 cases with elevation of cyclin D1 levels (13 and 14) were disomic (Figure 1I). None of the cases showed an 11q13 amplification.

***Histologic findings and immunohistochemistry***

Histologically, 39 cases (81%) were composed of well-differentiated plasma cells (Bartl grade I) and 9 cases (19%) were moderately differentiated (Bartl grade II).17 Ten cases revealed lymphoplasmacytoid differentiation (Table 2). Cyclin D1 expression was observed in 18 cases (37.5%). Strong, homogenous nuclear staining in the majority of tumor cells (> 80%, +++) was observed in 11 of 18 cases (Figure 3A). Intermediate cyclin D1 protein expression (20%-50%, ++) was observed in 4 cases (Figure 3B), and low levels of protein expression (10%-20%, +) were observed in 3 cases (Figure 3C). Cases 19, 21, and 24 were considered negative (Figure 3D); however, rare bona fide plasma cells were positive for cyclin D1. Four of 48 cases (8.3%) showed focal expression of CD20.

***Correlation between the expression of cyclin D1 mRNA and protein with the presence of 11q13 alterations***

The expression of cyclin D1 (mRNA and protein) together with the presence or absence of the t(11;14) translocation clearly defined 3 different groups. Group 1 included the 11 (23%) cases displaying high cyclin D1 mRNA levels. All 10 evaluable cases showed the t(11;14) translocation by segregation FISH and strong nuclear staining for cyclin D1 (+++; Table 2 and Figure 3A). Group 2 contained 15 (31%) cases with low to intermediate elevation of cyclin D1 mRNA and no evidence of the t(11;14) translocation.

Thirteen of these 15 cases showed polysomy by the locus-specific probe FISH. The 11q13 amplification was not identified in any of the cases. In contrast to the previous group, cyclin D1 protein expression varied from negative to (++) positive. The correlation between cyclin D1 mRNA and protein expression was generally good. Cases with low mRNA levels (cyclin D1/TBP ratio <10) tended to be negative or (+) positive for cyclin D1 protein expression, confirming our previous observations that real-time RT-PCR is a more sensitive method for the detection and quantitation of cyclin D1.[19] The association of 11q polysomy and low to intermediate cyclin D1 mRNA expression was statistically highly significant ($P < .0001$). Group 3 comprised the remaining 22 (46%) cases with normal or negative expression of cyclin D1(mRNA and protein) and no alteration of the 11q13 locus. Only in one case (case 35) was a polysomy for 11q identified by FISH.

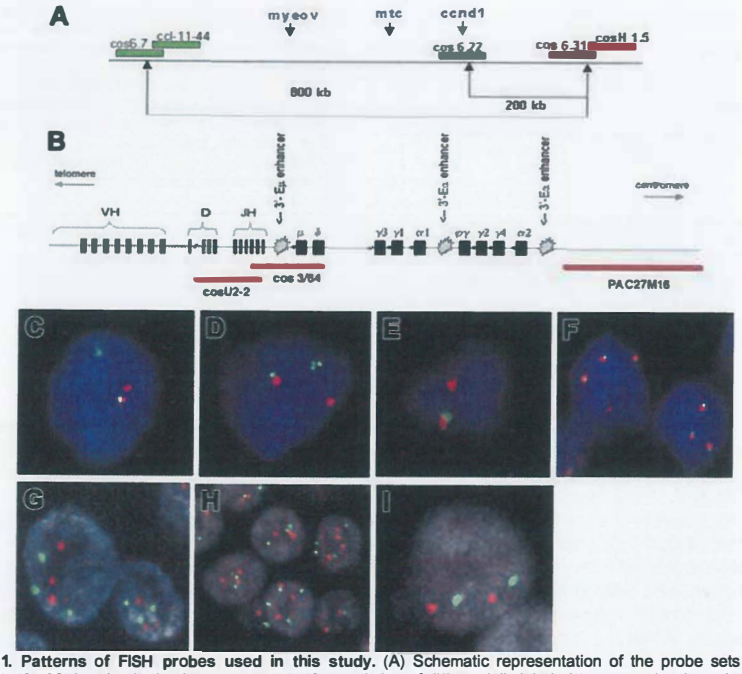


**Figure 1. Patterns of FISH probes used in this study.** (A) Schematic representation of the probe sets used for detection of the t(11;14)(q13;q32) breakpoint in chromosome 11q13, consisting of differentially labeled (green and red) pooled cosmids and P1- derived artificial chromosomes (PACs). (Adapted from Haralambieva et al[16] with permission.) (B) Schematic representation of the immunoglobulin heavy chain (IgH) gene locus and the IgH FISH probes used in the *CCND1* colocalization assay. Cosmid U2-2 covers JH and part of DH gene segments and cos3/64 covers the JH, Cμ, and Cδ gene segments, as described by Southern blotting analysis with segment-specific probes [22] and DNA fiber FISH mapping.[23] PAC clone PAC27M16 was mapped by DNA fiber FISH and located immediately 3' of the immunoglobulin Cα2 region (J. Guikema, E.S., and P.M.K., unpublished results, May 2003). (C-F) Segregation FISH analysis. Panels C and D both show a t(11;14)(q13;q32) translocation indicated by the presence of segregated red and green signals. Panel E shows a monoallelic translocation break with loss of the derivative chromosome der11, as indicated by the presence of a single red signal and the lack of a green signal. The colocalized signal (red/green) represents the normal allele. (F) Segregation FISH analysis showing increased copy numbers for the *CCND1* locus indicated by the presence of 3 colocalized signals. (G-I) Traditional interphase FISH analysis showing a trisomy (G), a polysomy (H), or a disomy (I). Original magnification X 630 for panels C-I.

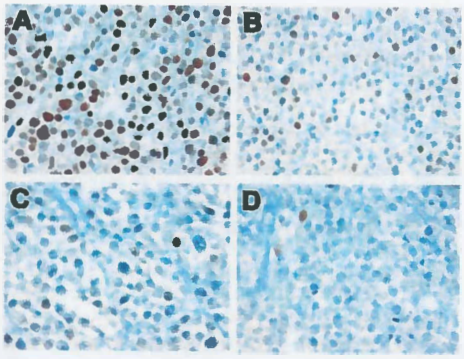


**Figure 3. Immunohistochemical analysis of cyclin D1 in 48 MMs.** (A) Representative case of MM group 1 showing strong, homogenous, cyclin D1 nuclear staining in the majority of tumor cells (> 80%, +++). Immunoperoxidase staining; original magnification X 400. (B) A representative case with intermediate cyclin D1 protein expression (++). Nuclear positivity is detected in 20% to 50% of the tumor cells. Note that the intensity of the staining varies from cell to cell. Immunoperoxidase staining; original magnification X 400. (C) MM with nuclear positivity in 10% to 20% of tumor cells (+) representing low levels of protein expression. Immunoperoxidase staining; original magnification X 640. (D) MM negative for cyclin D1. Note the positivity in endothelial cells used as internal control. Immunoperoxidase staining; original magnification X 640.

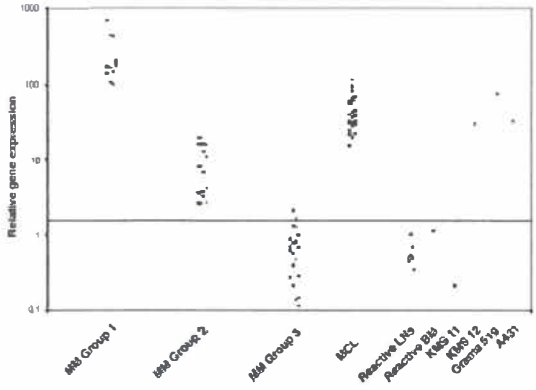


**Figure 2. QRT-PCR analysis of cyclin D1 mRNA levels in MMs.** QRT-PCR analysis of cyclin D1 was performed relative to the *TBP* housekeeping gene and results are depicted as ratio of cyclin D1/TBP transcript numbers. Three distinct groups of MMs with respect to cyclin D1 levels can be identified. Note for comparison the cyclin D1 levels in a group of 23 cases of mantle cell lymphoma (MCL), the KMS12 MM cell line harboring a t(11;14)(q13;q32) translocation, and the A431 squamous cell carcinoma cell line harboring a 11q13 amplification. The horizontal line (Tables 1-2). indicates the cutoff value for altered cyclin D1 expression as described in "Patients, materials, and methods."

## Discussion

In this study, we investigated cyclin D1 deregulation in a large series of primary MM cases and analyzed a potential correlation with the expression of MYEOV, a proposed oncogene lying in close vicinity to the t(11;14)(q13;q32) breakpoint region.[15] In addition, we investigated the influence of distinct translocation breakpoints and 11q13 copy numbers on the expression levels of cyclin D1 mRNA.

We found that high levels of cyclin D1, both at the mRNA and the protein level, occurred exclusively in the presence of a t(11;14)(q13;q32) translocation. There are only a few thorough studies, so far,[27,28] that correlate cyclin D1 mRNA levels with the presence of a t(11;14) translocation. This is largely due to technical obstacles, which have now been overcome by the availability of the real-time RT-PCR technique.[19,29] In this study, breakpoints in the 11q13 region were detected in 10 (21%) of the 47 evaluable cases by our segregation FISH approach. While the incidence of t(11;14) found in our study is similar to that described in 2 recent reports [12,30] (17% and 18%, respectively), we provide further evidence that the t(11;14) translocation is tightly connected with strong cyclin D1 overexpression, as shown by real-time RT-PCR (cyclin D1/TBP ratio > 95) and immunohistochemical analysis (+++; Table 2 and Figure 3A).

The strikingly high levels of cyclin D1 mRNA in MM cases with t(11;14) are in contrast with our previous findings in MCL,[19] where even though significantly elevated amounts of cyclin D1 mRNA were found in all samples (median cyclin D1/TBP ratio 31.9), they were well below the levels of most MM cases (Figure 2). The difference is most likely not due to the percentage of tumor cells since we enriched both MCL and MM by microdissection. There are 2 possible explanations for the higher cyclin D1 expression in MM. One is the presence of different breakpoints in the IgH locus at 14q32. In MCL, the break occurs in the JH region resulting in a direct juxtaposition of *CCDN1* to the 5'-Eμ enhancer and to the downstream 3'-Eα enhancers. The 14q32 breakpoint in MM is usually located in an IgH switch region resulting in the juxtaposition of *CCND1* onto the 3'-Eα enhancers. Accordingly, our colocalization FISH analysis revealed that in the MM cases with a t(11;14) translocation, *CCND1* expression is triggered by the 3'-Eα enhancers in at least 4 of the 7 analyzable cases. A second and more plausible explanation is that due to the higher levels of IgH transcription in MM, as opposed to MCL, the enhancers juxtaposed to the *CCND1* gene will trigger higher cyclin D1 transcription.

The second oncogene investigated in this study, *MYEOV*, lies in close proximity to the *CCND1* locus. For the first time, we demonstrate that MYEOV overexpression occurs in some primary MM cases with the t(11;14) translocation, presumably as a result of a reciprocal translocation.[15] This scenario, where both *MYEOV* and *CCND1* come under the separate control of different IgH enhancers seems to be a rare event, since high overexpression of MYEOV was found in only 2 of 10 translocated cases, paralleling the findings in MM cell lines.[15] Because the enhancers are joined to both *CCND1* and *MYEOV* in myelomas with a breakpoint in switch γ or switch α sites and since MYEOV expression is lost in most of these cases, our data strongly suggest that MMs do not favour MYEOV expression.

An interesting finding is the definition of a subgroup of MM patients with significant correlation between low to intermediate cyclin D1 mRNA overexpression and chromosome 11 polysomy (13/14; $P < .0001$). The presence of stable, intermediate cyclin D1 mRNA levels in several follow-up biopsies of 2 patients with chromosome 11 polysomy, taken up to 5 years apart, supports the notion that polysomy 11 is an essential factor related to cyclin D1 overexpression in these patients. Nevertheless, whether polysomy 11 per se directly or indirectly causes increased cyclin D1 expression in these cases remains to be clarified. The influence of the gene dosage effect through polysomy on cyclin D1 levels is difficult to explain since, until

influence of the gene dosage effect through polysomy on cyclin D1 levels is difficult to explain since, until now, it has been considered that cyclin D1 is normally not expressed in lymphoid cells. The frequency of polysomy 11 in our study (29%) is in the range of what has been reported previously (17%-37%).[27,30-33] However, the impact of this abnormality on cyclin D1 expression has not been thoroughly addressed. As shown in the present study, quantitative RT-PCR and not immunohistochemistry seems to be the method of choice to study this possible relationship: we found cyclin D1 staining in only 5 of 14 cases with polysomy 11, whereas 13 of 14 cases showed overexpression as determined by RT-PCR (Table 2). Similarly, in an immunohistochemical analysis by Pruneri et al,[30] only 2 of 13 cases with trisomy 11 showed cyclin D1 overexpression.

In a recent study, Soverini et al,[27] using real-time RT-PCR, showed that MM with trisomy 11 significantly overexpressed cyclin D1. However, in contrast to our data, where a clear distinction between patients harboring the t(11;14) translocation and those with trisomy 11 was observed, they found some overlap between the 2 groups. This discrepancy most probably reflects differences in the methodology. Whereas Soverini et al [27] used unpurified mononuclear cell fractions, we analyzed pure tumor cell populations obtained by microdissection.

There is still a small proportion of MM cases with intermediate overexpression of cyclin D1, lack of t(11;14), and no apparent molecular abnormalities of *CCND1* (2 of 15 cases, case 13 and 14), leaving open the possibility of additional deregulating mechanisms.

One potential explanation, DNA amplification in the 11q13 region, frequently observed in solid tumors,[34] was not found in any of our MM cases, as shown by FISH and quantitative PCR of cyclin D1 genomic DNA (data not shown). Other mechanisms include transcriptional regulatory mechanisms or insertion of any immunoglobulin regulatory elements, such as the 3'enhancer, as described for the U266 MM cell line.[35] In case of insertion, both breakpoints are located in the region flanked by cos6.7/cCl-11-44 and cos6.31/cosH1.5, and, therefore, this aberration will not be detected with the probe sets used in this study.

In contrast to previous studies,[12,36] it is now accepted that MM with the t(11;14)(q13;q32) translocation represents a unique subset of patients with a relatively favorable outcome.[37,38] Future studies examining prognosis of MM should take into consideration that cyclin D1 can be up-regulated independently of a t(11;14) translocation, as demonstrated in this study.

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# Chapter 9

## Summary and general discussion

Recent advances in our understanding of NHL biology as well as technical developments promote an expanding application of molecular techniques including FISH in research and clinical diagnostics. However, the application is hindered by the fact that most clinical materials are only available as formalin fixed and paraffin embedded tissue blocks in which the DNA is relatively degraded and heavily crosslinked. In consequence, most efforts were put in the (successful) development of PCR assays that can detect DNA sequence abnormalities in small DNA fragments that can be isolated from these tissue blocks. Another possibility is FISH but this application was also hampered by the cross linking and the masking effect of proteins on the DNA target. We and others [1,2] showed that introduction of a tissue pre-treatment step in the conventional FISH protocols is an effective solution for this technical problem. As a result multi- Yac constructs as well as small locus specific probes (PACs and cosmids) could be successfully applied for detection of chromosomal translocations in paraffin tissue sections (Chapter 2 and 3). All our subsequent FISH studies included in this thesis (Chapters 2, 3, 4, 5, 6 and 8) as well as the published reports by other groups [1,2] indicate that this approach is applicable to most of the fixed and routinely processed paraffin tumor samples,.

In Chapters 1, 2 and 3 we point out some intrinsic problems in the evaluation of FISH results in tissue sections, mostly related to the cutting artifacts and overlapping of cell nuclei. In addition, we suggested several algorithms for scoring of FISH signals, which have been tested on routine tissue sections (Chapter 2 and 3). Up to now, most investigators tend to adopt the scoring procedures from the cytogenetic laboratories. However, the performance of the FISH on routine tissue sections varies depending on the thickness of the sections, size of the cell nuclei, as well as the design of the FISH assay (segregation versus co-localization FISH). In addition, the malignant lymphoma clone often is only focally represented in the section and, in addition, it is often admixed with varying numbers of non-malignant cells that harbor normal FISH signal patterns. Therefore, various aspects of tissue sectioning and histopathological composition of the sample should be taken in consideration when employing FISH on paraffin tissue sections.

Methodologically, the FISH technique is restricted to a targeted detection of an already known abnormality (numerical aberration, translocation or deletion) and the availability of appropriate probes also represents an obvious limitation. However, the current human genome sequencing projects as well as the availability of very well characterized large inserts (BAC and PAC) by Internet resources, now enable the generation of an almost indefinite number of FISH probes for the identification of genetic abnormalities in lymphoid neoplasias. In parallel, the number of commercially available FISH probes is also steadily rising.

Interestingly, this expanding number of applications of the FISH technique in the routine pathology setting confronts the pathologists with new responsibilities that demand additional knowledge of molecular cytogenetics and collaboration with molecular biology experts. The individual performance of each FISH assay (commercially available or home made) needs to be carefully tested in the pathology laboratory before application in clinical diagnostics. In this line, in Chapter 4 we present the analysis of a newly generated FISH assay for the identification of chromosomal breakpoints in the 8q24 region in and nearby *MYC* gene. This study comprised the detection of *MYC* translocation breakpoints in a large series of lymphomas and cell lines with a cytogenetically proven 8q24 breakpoint and the subsequent testing of Burkitt lymphomas from three different geographic regions (Equatorial Africa, South America and Europe), in context to morphology, immunophenotype and EBV status.

Another important question is how to incorporate the generated molecular genetic data in the contemporary diagnostic setting: (1) are they specific as well as sensitive? (2) are they defining criteria for classification?, and (3) for which disease entities?, or (4) do they represent only additional information that should be interpreted in the context of the other data available? The wide application of methods like FISH in clinical pathology will certainly generate a large number of molecular data that should be closely correlated to the morphology, immunophenotype and clinical outcome. In consequence, the high scale application of such assays might probably revise our understanding or at the end even change our definition of certain disorders.

With respect to specificity of individual markers, it is already known that certain markers may be highly characteristic for a certain entity but not always specific. For instance the translocation t(14;18) is detected in approximately 85% of all follicular lymphomas, but also in 15-20% of the de novo DLBCL.[3,4]

The dilemma how to use these new markers is extensively discussed in Chapter 5, which encloses our data on a series of adult 'grey zone' lymphomas encompassing a spectrum of histomorphologies

between Burkitt lymphoma and diffuse large B-cell lymphoma, a notoriously difficult area in hematopathology. Obviously, the conventionally used panel of immunohistochemical markers (Ki67, bcl2 and CD10) helped the morphological diagnosis but was not able to solve the diagnostic dilemma in this field. In our study we made an attempt to improve further the histopathological distinction by introduction of FISH for *MYC*, *BCL2* and *BCL6* breakpoints. All data were compared to those obtained from a set of 'typical' pediatric Burkitt lymphomas. In keeping with the WHO recommendations [5] and our data in Chapter 4, the results indicate that due to its high sensitivity, FISH for detection of *MYC* breakpoints (or an equally sensitive method) should indeed be used as a prerequisite to the diagnosis of Burkitt lymphoma. However, as expected from literature data [6,7,8], this marker alone had a relatively low specificity and was not able to delineate any specific subset within this group of grey-zone lymphomas. Only some improvement in the recognition of a certain clinicopathologic entity was obtained by the combination with the immunohistochemical markers Ki-67, CD10 and bcl2. FISH for the detection of *BCL2* and *BCL6* breakpoints appeared less helpful, since breakpoints in these genes were less frequent than anticipated. However some cases that carry double translocation were identified and they mostly showed an abnormal phenotype. Therefore, we concluded that the combination of immunohistochemistry with FISH for *MYC* breakpoint detection certainly improved the diagnostic process, however, even this rather elaborate set of markers is insufficient to delineate any solid entity of adult Burkitt lymphoma. Likely, novel data obtained from micro-array gene expression studies and perhaps also genome wide genetic studies, should be awaited before any final conclusions can be drawn.

Certainly, the presence of a *MYC* breakpoint as detected in 100% of the pediatric and 57% of the grey zone lymphomas as described in chapter 5, confers a distinct morphology and immunophenotype. However, in the series of grey zone lymphomas studied by us a *MYC* breakpoint alone did not recognize further any distinct lymphoma subgroup. One explanation is that *MYC* might be deregulated in far more cases than identified by us since other genetic mechanisms (e.g. mutations in essential regulatory sites of *MYC* [9]) or epigenetic factors (demethylation and histone acetylation) may result in the same functional endpoint. On the other hand, this possibility is implausible since more than 95% of all regular BL as studied in chapter 4 and all 10 pediatric BL studied in chapter 5 carried an easily detectable *MYC* breakpoint. Preliminary results from array derived gene expression data indicate that BL is characterized by a combination of gene expression patterns, each involving of large number of different genes (EJ Boerma, L Staudt, NCI, NIH, Bethesda). Whereas *MYC* represents an important key regulator of various aspects of the cell cycle, and consequently in the control of numerous recognized downstream genes, this *MYC* associated gene expression pattern is not sufficient to characterize BL. For instance, in contrast to DLBCL with a MYC breakpoint and overexpression of *MYC*-related genes, BL show downregulation of NF-kB and many NF-kB related genes. Therefore, it will be important to define which other key regulators and concomitant gene expression patterns besides *MYC* and the numerous *MYC* related genes are characteristic for BL.

Eight cases from these series (3 BL and 5 DLBC, Chapter 5) carried a combination of *MYC* and *BCL2* or *BCL6* breakpoints. In tumors with a double translocation involving *BCL2* and *MYC*, it has been proven that the translocation involving *BCL2* is the first hit (generated during VDJ recombination in a precursor B-cell), and the alteration affecting *MYC* - the second one (mostly generated during IgH hypermutation or class switch recombination).[6] This suggests that *MYC* alteration and deregulation confers a certain (immuno-) morphology, even if it occurs as a secondary hit. Most tumors with a concomitant *MYC* and *BCL2* breakpoint represent a very aggressive disease with a poor clinical outcome which is in agreement with the fact that most tumors develop through transformation of a clinically overt follicular lymphoma.[6,7] Since according to the current view, BL is a primary disease,[5,10] probably such cases should not be classified as BL but separated.

Lymphomas with a combination of a *MYC* and *BCL6* breakpoint have been reported occasionally, [8, 11] however, the clinical and biological significance of these alterations has not been established. It is not clear which rearrangement occurs first and whether this combination confers a synergistic growth advantage on the lymphoma cells. One salient feature of our two BL cases with this dual translocation was the overexpression of the bcl2 protein, a feature also seen in cases with a double translocation involving *MYC* and *BCL*-2 (Chapter 5). Although the selected three genes (*MYC*, *BCL2* and *BCL6)* are the most commonly involved in the pathogenesis of these lymphomas, they by far do not represent the molecular profile of the entities. Cytogenetic studies on BL suggest that the t(8;14) or variant translocations are the sole abnormality in almost half of the pediatric BL cases and that adult BL cases have more complex karyotypes with many additional alterations (http://cgap.nci.nih.gov/Chromosomes/CytSearchForm). This raises the question whether the category of adult BL might contain a proportion of transformed tumors not recognized by the more conventional means such as morphology, immunophenotype and the *MYC* breakpoint analysis. Certainly, more genome-wide genetic approaches such as classical karyotyping, CGH and array-CGH in combination with gene expression studies are needed to establish the biological relationship between BL and DLBCL.

In chapter 7, we gave a brief introduction to myeloma. Our study on a large series of multiple myelomas, presented in chapter 8, in keeping with published data [12,13,14], indicate that the 11q13 breakpoint is associated with high cyclin D1 RNA and protein expression. However, we also identified a subgroup of MM

that lack an 11q13 breakpoint and still steadily express cyclin D1 RNA at medium levels. Correspondingly, using immunohistochemistry, these samples were labeled for cyclin D1 protein in varying percentages of tumor cells and with heterogeneous intensity. Our data indicate an association with a trisomy 11 in these MM cases with an intermediate expression. The insertion of IgH enhancer sequences into the 11q13 locus has been reported to upregulate cyclin D1 expression in a MM cell line.[15] Although we did not exclude a possible insertion in our MM series, this activation mechanism seems very unlikely. The high levels of cyclin D1 RNA in all cases with 11q13/cyclin D1 breakpoints (e.g. cyclin D1 deregulation upon the juxtaposition to IgH enhancer) distinguish them sharply from the other samples.

In consequence, juxtaposition of enhancer sequences should trigger a similar very high level of cyclin D1 RNA expression, but this is not seen in our MM patients. Solely a gene dosage effect by trisomy 11 is also an unlikely explanation since normal B cells and mature plasma cells entirely lack cyclin D1 expression. Alternatively epigenetic factors such as demethylation or histone acetylation might play a role as well. In cases without any breakpoint or trisomy 11 such a yet undefined epigenetic mechanism might be responsible for the basic cyclin D1 upregulation in some cases of MM, and this basic upregulation might be incremented to medium levels in MM cases with trisomy 11. In spite of all recent research, it is still not clear how cyclin D1 contributes to the pathogenesis of MM, however, apparently certain levels of cyclin D1 are necessary for the maintenance of the neoplastic clone.[16] In addition, it should be noted that MM patients with an 11q13 translocation breakpoint and concomitant cyclin D1 deregulation are currently considered to represent a biologically and clinically defined group.[14,16] The relevance of the intermediately elevated cyclin D1 RNA levels in the absence of an 11q13 breakpoint remains to be identified.

In keeping with previous reports on MM cell lines, [17] our study showed that in a subset of MM patients the t(11;14) translocation results in concurrent deregulation of *MYEOV*, another 11q13 oncogene (Chapter 8). In contrast to cyclin D1, the expression levels of *MYEOV* were not strictly linked to the 11q13 breakpoint status, however, the lack of sufficient information on *MYEOV* expression in normal and reactive hematopoietic tissues limits the significance of our data. In addition, similar to some other MM translocations with activation of two different genes, in a subset of MM cases the der14 with the translocated *MYEOV* gene is lost (Chapter 8). This suggests that deregulation of cyclin D1 and not *MYEOV* is the critical oncogenic event triggered by the t(11;14)(q13;q32) translocation in MM patients.

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## Nederlandse samenvatting

De recente ontwikkelingen in het onderzoek naar de biologie van Non Hodgkin lymfomen (NHL) en de gelijktijdige technische ontwikkelingen hebben de toepassing van moleculaire technieken zoals fluorescente in situ hybridisatie (FISH) in het wetenschappelijk onderzoek en de klinische diagnostiek in een stroomversnelling gebracht. Een van de beperkingen is echter dat heel veel diagnostisch tumor materiaal in formaline gefixeerd wordt waardoor het DNA o.a. "gecross-linked" en afgebroken wordt en niet meer toegankelijk is voor een aantal van deze technieken. Om dit te omzeilen werd in eerste instantie veel aandacht besteed aan de ontwikkeling van op paraffine materiaal toepasbare PCR technieken (waarbij kleine stukken DNA vermenigvuldigd en zichtbaar gemaakt kunnen worden). Het alternatief voor een aantal vraagstellingen is FISH, maar ook deze techniek heeft te leiden onder de cross-linking van DNA en maskering door aanwezige eiwitten. Wij en anderen hebben speciale voorbehandelingsmethodes ontwikkeld die het mogelijk maken deze techniek op routine paraffine coupes van formaline gefixeerd materiaal toe te passen. Met deze methode konden we heel grote (gist-artificiële chromosomen – YAC) tot heel kleine gen-specifieke (cosmides) probes gebruiken. De laatste probes zijn uitermate geschikt voor de detectie van specifieke chromosomale translocaties (hoofdstuk 2 en 3). In de opeenvolgende hoofdstukken hebben we steeds van deze voorbehandelingsmethode en FISH technieken gebruik gemaakt.

Net als PCR, en in tegenstelling tot klassieke cytogenetica heeft FISH een beperkte toepassing voor alleen van tevoren gedefinieerde DNA targets. Echter de nu aanwezige en beschikbare kennis ten aanzien van vrijwel de complete DNA structuur en volgorde van baseparen ("het humane genoom"), en de enorme hoeveelheid inmiddels (deels commercieel) beschikbare DNA probes heeft de toepassing van FISH sterk vereenvoudigd.

In de **hoofdstukken 1, 2 en 3** behandelden we de problemen die verbonden zijn met de beoordeling van FISH signalen in weefselcoupes waarbij het intrinsieke probleem bestaat dat celkernen, en daarmee ook de FISH signalen in de kern, door de aansnijding incompleet zijn. De tot nu toe veel gebruikte scoringsmethodes uit de klassieke cytogenetica zijn door de aansnijdingsartefacten in coupes niet adequaat. Daarbij hebben we verschillende algoritmes gevonden die geschikt zijn om FISH signalen te kunnen kwantificeren en interpreteren (**hoofdstuk 2 en 3**).

De vlucht van de moleculaire technieken zoals FISH confronteert de klinisch patholoog met een nieuwe verantwoordelijkheid: hoe dient deze de technieken toe te passen en te interpreteren, en welke kwaliteitseisen moeten aan de toepassing ervan worden verbonden? In **hoofdstuk 4** geven we een voorbeeld hoe wij de door ons ontwikkelde FISH test voor de detectie van breuken op chromosoom 8q24 ter plaatse van het *MYC* gen hebben gevalideerd. Dit breukpunt komt met name voor in het Burkitt lymfoom (>95% van alle tumoren), een zeldzame maar zeer agressieve vorm van NHL. De aanwezigheid van de chromosomale breuk is een voorwaarde om de diagnose te kunnen stellen. Patiënten met deze vorm van NHL dienen indien ook maar enigszins mogelijk met speciale agressieve chemotherapie schema's behandeld te worden. De validatie bestond uit een studie waarin we eerst de technische sensitiviteit en specificiteit in een grote serie van tumor samples met bewezen chromosomale 8q24 afwijking alsmede controle samples hebben bepaald. Vervolgende besloten wij de performance in de dagelijkse praktijk te testen in een grote serie klinische samples waaronder Burkitt lymfomen uit equatoriaal Afrika en Zuid-Amerika (twee gebieden waar dit lymfoom endemisch voorkomt) en Nederland. Uit deze analyse bleek dat de FISH test een hoge specificiteit en sensitiviteit had.

Daarna resteerde de vraag in hoeverre de FISH test ook behulpzaam is in de dagelijkse praktijk van de patholoog, met name in hoeverre deze behulpzaam is in de differentiaal diagnostiek tussen verschillende NHL subtypen, en in hoeverre de resultaten op zichzelf staan of alleen gebruikt kunnen worden in combinatie met andere aanvullende technieken zoals immuunhistochemie. Inmiddels weten we voor veel van dergelijke markers dat een bepaalde afwijking weliswaar kenmerkend, maar niet specifiek hoeft te zijn voor een bepaald tumor type.

Het dilemma hoe deze markers in de afwegingen van de patholoog kunnen worden geïntegreerd, wordt in **hoofdstuk 5** besproken. In dit hoofdstuk hebben we een groot aantal grijze-zone lymfomen onderzocht, dwz lymfomen die lijken op zowel het Burkitt lymfoom als diffuus grootcellige B-cel lymfoom. Dit probleem doet zich vooral voor bij volwassen patiënten en vormt een notoir dilemma voor ook zeer ervaren hematopathologen. Conventionele immuunhistochemie (analyse van het Ki-67 eiwit als maat voor proliferatie, het anti-apoptotisch bcl2 eiwit het eiwit CD10, een eiwit met grotendeels onbekende functie) is behulpzaam maar lost het differentiaal-diagnostisch probleem tussen beide typen NHL niet op. In hoofdstuk 5 probeerden wij het onderscheid te verbeteren door aan deze reeds bekende eiwit markers enkele genetische markers die met FISH bepaald kunnen worden, toe te voegen. Dat waren de detectie van chromosomale breukpunten in 8q24/*MYC*, 3q27/*BCL6* en 18q21/*BCL2*. In een klassiek Burkitt lymfoom verwacht men in 95% van alle gevallen een breuk in 8q24/*MYC* maar niet in de andere gebieden. Daarentegen komen breuken in de andere gebieden vaak (respectievelijk 35% en 20%) voor in de klassieke

diffuus grootcellige B-cel lymfomen. Alle gegevens die we verkregen van een serie van 74 grijze-zone lymfomen werden vergeleken met die van een serie van klassieke Burkitt lymfomen bij kinderen. Toepassing van alleen de FISH voor 8q24/*MYC* bleek absoluut onvoldoende toevoegende warde te hebben: terwijl de sensitiviteit hoog was, was de specificiteit laar: ongeveer eenderde van de grijze-zone lymfomen die wat betreft de meer conventionele markers niet voldeden aan de criteria van een Burkitt lymfoom bezaten toch een 8q24/MYC breukpunt. Uit deze studie bleek dat de gecombineerde toepassing van immuunhistochemie en FISH voor leifst alle drie de genetische afwijkingen vooralsnog absoluut noodzakelijk is om de diagnose Burkitt lymfoom met enige zekerheid te kunnen stellen, maar dat ook deze gecombineerde aanpak niet optimaal is. Op dit moment zijn dan ook diverse onderzoeksgroepen bezig om de genexpressie patronen en genetische afwijkingen van deze lymfomen veel uitgebreider in kaart te brengen. Dit gebeurt o.a. met technieken als gen expressie arrays en DNA 'comparative genomic hybridization' (CGH) arrays. Waarschijnlijk moeten we eerst de resultaten hiervan afwachten voordat we dit diagnostische probleem kunnen oplossen.

Het *MYC* gen op chromosoom 8q24, is een uitermate belangrijk gen in de regulatie van o.a. de cel cyclus van delende cellen. Activatie van *MYC* leidt tot de activatie van duizenden andere genen. Een interessante wetenschappelijke vraag is daarom in hoeverre de activatie van *MYC* betrokken is in het fenotype en gedrag van Burkitt lymfoom cellen en ook de lymfoom cellen van deze grijze-zone lymfomen.

Acht lymfomen uit de serie bevatten meerdere translocaties. Uit de literatuur was reeds bekend dat de combinatie van een 8q24/*MYC* en 18q21/*BCL2* breukpunt in een tumor geassocieerd is met een Burkitt lymfoom morfologie; tumoren met deze afwijkingen gedragen zich vaak zeer agressief en de meeste van deze patiënten overlijden door progressie van de ziekte. Ook enkele tumoren met een gecombineerde 8q24/*MYC* en 3q27/*BCL6* breuk zijn wel beschreven, maar de klinische consequenties hiervan zijn tot op heden onbekend.

In **hoofdstuk 6** worden 4 Burkitt lymfomen met een opmerkelijke morfologie beschreven. Alle vier tumoren werden gekarakteriseerd door de aanwezigheid van niet necrotiserende granulomen tussen de tumorcellen. Bij enkele patiënten hadden deze granulomen de patholoog misleid wat betreft de diagnose Burkitt lymfoom. Wij vonden dat alle tumoren naast het klassiek fenotype en de chromosomale breuk 8q24/*MYC* ook het Epstein Barr Virus (EBV) bevatten. Dit werd aangetoond met een andere in situ hybridisatie techniek, namelijk ISH voor de EBER RNA moleculen. In dit hoofdstuk speculeerden wij ook over de pathogenese van deze granulomen.

In de **hoofdstukken 7 en 8** bestudeerden wij de aanwezigheid en betekenis van een andere chromosomale breuk, namelijk die in 11q13/*CCND1* bij de hematologische maligniteit multipel myeloom (ziekte van Kahler). Dit betreft een tumor van rijpe plasmacellen in het beenmerg en de botten. In deze tumor komen diverse chromosomale translocaties voor die met elkaar gemeen hebben dat een breukpunt in de constante delen van het immunoglobuline gen complex op chromosoom 14 ligt. Een van de partners is cycline D1 op chromosoom 11q13, ook betrokken bij >95% van het mantelcel lymfoom. Dit leidt tot de translocatie t(11;14)(q13;q32). In **hoofdstuk 7** wordt een overzicht gegeven van deze chromosomale afwijkingen in multipel myeloom. **In hoofdstuk 8** onderzochten wij de relatie tussen deze chromosomale translocatie en overexpressie van cycline D1 in een grote serie multipel myelomen. FISH, kwantitatieve RT-PCR en immunohistochemie experimenten lieten zien dat de translocatie gepaard gaat met een hoge cycline D1 RNA en eiwit expressie in de tumorcellen. Daarnaast was er een groep myelomen met matig hoge expressie zonder translocatie maar met een trisomie van chromosoom 11. Mogelijk draagt "gene-dosage" bij aan de overexpressie in deze myelomen. Daamaast was er een categorie myelomen met lage of geen expressie. Deze toonden geen translocatie of trisomie 11. Mogelijk spelen epigenetische factoren zoals demethylatie of acetylatie een rol bij de cycline D1 expressie in deze myelomen zonder genetische afwijkingen. Myelomen met een translocatie t(11;14) vertegenwoordigen waarschijnlijk een aparte groep; patiënten met deze myelomen hebben een wat betere prognose dan andere myeloom patiënten.

## Abbreviations

| | |
|---|---|
| ABC | activated B cells |
| ALK | anaplastic large cell kinase |
| ALL | acute lymphoblastic leukemia |
| BAC | bacterial artificial chromosome |
| BCR | B cell receptor |
| BL | Burkitt lymphoma |
| aBL | atypical Burkitt lymphoma |
| eBL | endemic Burkitt lymphoma |
| sBL | sporadic Burkitt lymphoma |
| BLL | Burkitt-like lymphoma |
| CGH | comparative genomic hybridization |
| CLL | chronic lymphocytic leukemia |
| CSR | class switch recombination |
| DLBCL | diffuse large cell lymphoma |
| EBV | Epstein Barr virus |
| IG | immunoglobulin |
| FGFR3 | fibroblast growth factor receptor 3 |
| FICTION | Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms |
| FISH | fluorescence *in situ* hybridization |
| FL | follicular lymphoma |
| GC | germinal center |
| GCB | germinal center B cell |
| HIV | human immunodeficiency virus |
| HCL | hairy cell leukemia |
| HMCL | human myeloma cell lines |
| IGH | immunoglobulin heavy chain |
| IGL | immunoglobulin light chain |
| M-FISH | multicolor fluorescence *in situ* hybridization |
| MALT | mucosa associated lymphoid tissue |
| MCL | mantle cell lymphoma |
| MGUS | monoclonal gammopathy of undetermined significance |
| MM | multiple myeloma |
| MZL | marginal zone lymphoma |
| NHL | non Hodgkin's lymphoma |
| NF-κB | nuclear factor –κappa B |
| PAC | P1 artificial chromosome |
| PCR | polymerase chain reaction |
| PC | plasma cell |
| PFGE | pulsed-field gel electrophoresis |
| SKY | spectral karyotyping |
| SMM | smoldering multiple myeloma |
| WHO | World Health Organization |
| YAC | yeast artificial chromosome |